ANALYSIS OF 5 GENES UNIQUE TO EQUINE HERPESVIRUS 1 AND CHARACTERISATION OF THE PRODUCTS OF GENES 67 AND 71

BY

YI SUN

A Thesis Presented for the Degree of Doctor of Philosophy

in

The Faculty of Medicine University of Glasgow

ProQuest Number: 13818903

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 13818903

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to acknowledge the generous sponsorship of The Equine Virology Research Foundation and to thank Professor J. H. Subak-Sharpe for providing the excellent research facilities in the Institute of Virology.

I am particularly grateful to my supervisor, Professor M. S. Brown for her scientific guidance throughout this work and her valuable criticism of the manuscript, and many thanks to Dr. A. R. MacLean for his scientific advice and technique help as well as criticism of the manuscript.

I would like also to thank to Drs. A. J. Davison and E. A. R. Telford for providing equine herpesvirus 1 virus, unpublished data and technical help, and to Drs. H. J. Field and T Fitzmaurice for providing unpublished data, and to Drs. C. MacLean, and D. Darrick for their technical help, and to N. D. Stow for providing the plasmid, pCMV10, and to Mr. J. D. Aitken for carrying out the electron microscope work.

I would like to thank all the members of Labs 209 and 210 and all staff in the Institute of Virology for producing a good scientific environment and research facilities in the Institute.

I would like to thank my parents for their interest in my work and in valuable encouragement and support throughout the years.

Finally, words can not express the depth of the gratitude I feel towards my wife, Amina and my son, David, who loyally supported me throughout the years. Without their constitute love, interest and encouragement this work would never have been completed.

During this work the author was supported by a Equine Virology Research Foundation. Unless otherwise specified, all the results reported were obtained by theauthor'sown efforts.

Yi Sun (August 1996).

ABSTRACT

Equine herpesvirus 1 (EHV1), a member of the alphaherpesvirinae, is a significant viral pathogen of horses causing a variety of clinical problems including respiratory disease, abortion and neurological disorders. The complete DNA sequence of EHV1 strain Ab4, a pathogenic UK isolate has been determined. There are 81 open reading frames (ORF) predicted from the DNA sequence. Most of these predicted gene products have not been identified and their functions in the virus life cycle are not known. Among these putative genes, five 1, 2, 67, 71 and 75 had no detectable homologues in HSV1 and VZV, and appeared to be unique to EHV1 (Telford *et al.*, 1992). This feature suggested that these genes may have specific roles in the virus life cycle in the horse. The research presented in this thesis has concentrated on investigating the role of the five genes in the virus life cycle. The project included three parts: (1) Construction and characterisation of deletion mutants in the target genes. For construction of deletion mutants the majority of the encoding sequence of each target gene was removed and replaced by the *E. coli lacZ* gene as a marker gene. The isolation of viable mutants in the five target genes demonstrated that none of these genes were essential in the virus life cycle *in vitro*. Their growth characteristics, host range and temperature sensitivity in tissue culture were indistinguishable from those of wild type virus with the exception of the gene 71 deletion mutant (ED71). ED71 grows less well than wild-type virus and its revertant, produces smaller plaques and has an obviously higher particle/pfu ratio of progeny virus than the wild-type virus and revertant *in vitro*. (2) Identification and characterisation of the target gene products. To raise specific antisera the genes were separately expressed as ß-galactosidase fusion proteins in *E.coli*. These consisted of amino acids 4-202 of the putative gene 1 protein, amino acid sequence 2-205 of the putative gene 2 protein, amino acids 12-260 of the putative gene 67 protein, amino acids 434-797 of the putative gene 71 protein and amino acids 14-130 of the putative gene 75 protein. Two antisera, anti-67 and anti-71, have been raised against the gene 67 and gene 71 fusion proteins in rabbits. Use of the deletion mutants in conjunction with the antisera,

allowed the unambiguous identification of the protein products of genes 67 and 71. Anti-67 serum specifically recognised a 36KD M_r polypeptide in infected cell extracts which was absent from mock and mutant ED67 infected cell extracts and was not recognised by the pre-immune serum. In the lytic replication cycle, the protein is regulated as an early polypeptide and modified as a phosphoprotein. With immunofluorescence and cellular fractionation experiments, the protein has been shown to be associated with intracellular membranes and distributes as novel ribbon or filament like structures, concentrated in a perinuclear location within the cytoplasm of infected cells. The protein does not co-localise with either tubulin, actin or Golgi bodies. However, the precise localisation of the gene 67 protein within cells is unclear. The gene 67 protein is also a component of virions and is located in the nucleocapsid/tegument. Anti-71 serum, specifically recognises a 192KD M_r infected-cell polypeptide. This protein is absent from mock and mutant ED71 infected cell extracts and is not recognised by pre-immune serum. This result confirmed that the 192KD polypeptide was encoded by EHV1 gene 71. Gene 71 is regulated as a leaky late gene during EHV1 lytic infection. The 192 KD gene 71 protein is associated with cellular membranes and is also a component of the virion envelope with a M_r > 200KD. Post-translational modification experiments showed that the gene 71 protein was sensitive to tunicamycin and monensin; the M_r of the protein was greatly reduced by deglycosylation with exoglycanases, which demonstrated that the gene 71 protein was post-translationally modified as a glycoprotein with limited N and heavy O-linked glycosylation. These features of the gene 71 protein indicate that the protein is a class I membrane glycoprotein which is consistent with the features of the gene 71 protein predicted from sequence analysis (Telford *et al.*, 1992a).

During the course of this study, it became clear that the gene 71 protein had very similar properties to that of gp300, a EHV1 glycoprotein, which had been characterised as being heavily O-linked with a M_r over 400 KD in SDS-PAGE linked by DATD. gp300 had been previously found to be encoded by EHV1 gene 28 (Whittaker *et al.*, 1990; 1992a). To determine the relationship between the gene 71 protein and gp300, a comparative experiment was carried out with anti-71 and a monoclonal antibody, P19, which specifically recognised

gp300 (Whittaker, *et al.*, 1992a). The comparative data shows that gp300 and the gene 71 protein have the same molecular weights under the same gel conditions and are both absent from ED71 infected cell extracts, but restored in ED71 revertant infected cell extracts. This data clearly confirmed that gp300 was encoded by EHV1 gene 71. Its previous designation as the product of gene 28 was incorrect. ED71 has been found to be impaired in growth *in vitro* and produces smaller plaques as well as having a high particle/pfu ratio. Since the revertant of ED71 grows identically to wild-type virus, the deletion of gene 71 is responsible for the phenotype of ED71. As the gene 71 protein has been characterised as a class I membrane glycoprotein, the results suggested that the gene 71 protein may play a role in virus entry into cells, egress from cells or transmission from cell to cell. (3) To functionally analyse the role of the gene 71 protein, ED71 was characterised in terms of efficiency of entry into cells *in vitro*, egress from cells and transmission from cell to cell, compared with wild-type and revertant virus. The results showed that the deletion of gene 71 affected viral nucleocapsid envelopment and resulted in greatly decreased egress of infectious virus progeny from cells. ED71 also displays a defect in entry into cells. These defects have a consequential effect on efficiency of the virus transmission via release and readsorption to uninfected cells.

ABBREVIATIONS

APS	ammonium persulphate
ATP	adenosine triphosphate
BHK21/C13	baby hamster kidney cells batch 21 clone 13
BHV	bovine herpesvirus
bp	base pairs
BSA	bovine serum albumin
°C	degree centigrade
CAT	chloramphenicol acetlytransferase
CCV	channel catfish virus
CHO	chinese hamster ovary
c.p.e.	cytopathic effect
dATP	2'-deoxyadenosine-5'-triphosphate
DATD	N'N'-diallitratardiamide
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
ddATP	2',3'-dideoxyadenosine-triphosphate
ddCTP	2',3'-dideoxycytidine-triphosphate
ddGTP	2',3'-dideoxyguanosine-triphosphate
ddTTP	2',3'-dideoxythymidine-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
DTT	dithiothreitol
EBV	Epstien-Barr virus
E.coli	*Eschericia coli*
EDTA	ethylenediaminetetra acetic acid

EHV1	equine herpesvirus 1
EHV2	equine herpesvirus 2
EHV3	equine herpesvirus 3
EHV4	equine herpesvirus 4
EHV5	equine herpesvirus 5
G	guanine
HBLV	human B-cell lymphotropic virus
HCMV	human cytomegalovirus
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HHV	human herpesvirus
HSV1	herpes simplex virus type 1
HSV2	herpes simplex virus type 2
HVA	herpesvirus ateles
HVS	herpesvirus saimiri
ICP	infected cell polypeptide
IE	immediate early
Ig	immunoglobulin
IPTG	isopropyl-ß-D-thiogalactoside
IR_L	internal long repeat
IR_S	internal short repeat
k	kilo (ie. 10^3)
kbp	kilo base pairs
L	long segment
LAT	latency associated transcript
MDV	Marek's disease virus
mg	milligram
mM	millimolar
m.o.i.	multiplicity of infection
M_r	molecular weight

m.u.	map unit
ng	nanogram
np	nucleotide position
NP40	Nonidet P40
OD	optical density
ORF(s)	open reading frame (s)
oriL	origin of replication in U_L
oriS	origin of replication in U_S
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
p.f.u.	plaque forming units
p.i.	post-infection
PRV	pseudorabies virus
R1	large subunit of ribonucleotide reductase
R2	small subunit of ribonucleotide reductase
RE	restriction enzyme
RNA	ribonucleic acid
r.p.m.	revolution per minute
RR	ribonucleotide reductase
RT	room temperature
S	short segment
SDS	sodium dodecyl sulphate
T	thymidine
TEMED	N,N,N',N',-tetramethlyethylene diamine
Tris	tri (hydroxymethyl) aminomethane
TK	thymidine kinase
TR_L	long terminal repeat

TR_S	short terminal repeat
ts	temperature sensitive
µCi	microcurie
U_L	long unique
µl	microlitre
µM	micromolar
U_S	short unique
uv	ultraviolet
V	volts
Vmw	molecular weight in kilodaltons of HSV induced polypeptides
VP	virion protein
v/v	volume/volume (ratio)
VZV	varicella zoster virus
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)
wt	**wild type**

ONE AND THREE LETTER ABBREVIATIONS FOR AMINO ACIDS

Amino acids	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Isoleucine	Ile	I

CHAPTER 1. INTRODUCTION

1.1 GENERAL DESCRIPTION OF HERPESVIRUSES

To date eight different herpesviruses whose natural host is man have been identified: herpes simplex virus type 1(HSV1), herpes simplex virus type 2 (HSV2), varicella zoster virus (VZV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and human herpesviruses (HHV) types 6-8. They appear to be ubiquitous, and serological studies show that a large proportion of people throughout the world have been exposed to and may be latently infected with one or more herpesvirus. Clinical manifestations of herpesvirus infections range from inapparent infection to localised disease and fatal systemic illness. HSV1, HSV2 and VZV, alphaherpesviruses, are neurotropic viruses which typically cause mild primary infection of epithelial cells of the skin giving rise to vesicular lesions. HSV1 is typically associated with lesions of the mucocutaneous epithelial lining of the oral cavity (herpes labialis) and eyes (herpeskeratitis), while HSV2 is predominately associated with lesions of the genital tract (herpes genitalis) (Whitley, 1985). VZV results in the childhood disease known as chicken pox (varicella). HCMV, a beta-herpesvirus, tends to be species-specific *in vitro*. HCMV infection in normal adults is wide spread, but results in a benign and asymptomatic disease. However, the disease can be disseminated and severe in children or immunosuppressed patients. EBV, a gammaherpesvirus, is associated with Burkitts lymphoma (a B-cell malignancy) and in South East Asia, with nasopharyngeal carcinoma (an epithelial cell malignancy). HHV6, (Ablashi *et al*., 1987) was found to be wide spread in adults and segregated into two groups (HHV6A and HHV6B) based on biological, immunological and molecular analyses. The only disease in which HHV6B has been implicated is exanthum subitum, a transient childhood illness (Yamanishi *et al.*, 1988). HHV7 was isolated from peripheral blood lymphocytes (PBLs) of a healthy individual in 1989 (Frenkel *et al.*, 1990). Since then HHV7 has been isolated from saliva of as many as 75% of healthy adults (Levy *et al.*, 1990) and analysis of the viral DNA indicates it differs from the other known herpesviruses- no disease has been associated with this virus. Very recently herpesvirus-like DNA sequences have been found associated with

various forms of Kaposi's sarcoma, but not normal tissue (Chang *et al.,* 1994). The DNA sequences of Kaposi's sarcoma herpesvirus (KSHV) or HHV8 are highly homologous to genes of HSV and EBV. A high prevalence of KSHV in endemic as well as AIDS-associated Kaposi's sarcoma was found suggesting that KSHV might be associated with immunodeficiency in general, and indeed KSHV has been associated with lesions other than Kaposi's sarcoma in non-AIDS immunocompromised patients (Rady *et al.,* 1995).

Herpesviruses also cause several diseases in animals. Equine herpesvirus 1 (EHV1) is the major cause of abortion and respiratory disease in horses. Pseudorabies virus (PRV) causes Aujewsky's disease in swine. Bovine herpesvirus 1 (BHV1) is the etiologic agent of infectious bovine rhinotracheitis. Lucké virus causes renal adenocarcinomas in the frog (Gravell, 1971). Marek's disease virus (MDV) induces transformation of lymphoblastoid cells in chickens (Churchill and Biggs, 1968).

In general, following primary infection, alphaherpesviruses can persist in neuronal cells in a latent form and may give rise to periodic recurrence of the disease during the life time of the host. EBV is also capable of establishing latency in lymphocytes. It efficiently immortalizes B-lymphocytes *in vitro* (Dambaugh *et al.,* 1986). The sites and essential features of betaherpesvirus latency are unknown.

1.2. CLASSIFICATION OF HERPESVIRUSES

Members of the family herpesviridae have been classified on the basis of morphology and cytopathology (Matthews, 1979, 1982). All members possess a large double-stranded DNA genome present within an enveloped particle (150-200 nm in diameter). The virion is composed of four distinct elements; the core, capsid, tegument and envelope. The core contains the genomic DNA (Booy *et. al.*, 1991). It is surrounded by an icosahedral shaped capsid assembled from 162 capsomers of which 150 are hexomeric and 12 pentameric prisms (Wildy *et al.*, 1960, Schrag *et al.,* 1989). The amorphous material arranged around the nucleocapsid is known as the tegument. The envelope is the outermost structure of

virus and comprises a lipid bilayer derived from host cell nuclear and plasma membranes from which protrude numerous viral-encoded glycoprotein spikes of approximately 8 nm in length (Heine et al 1972). Herpesviruses differ widely in their pathogenic potential but share important biological properties. Most notable is the ability, after primary infection, to establish latent infections throughout the lifetime of the host. Other biological features in common to all herpesviruses include : (1) Viral DNA is synthesised and capsid assembly takes place in the nucleus and (2) They encode a variety of virus-specified enzymes, particularly those involved in nucleic acid metabolism and DNA replication.

1.2.1. Subclassification on the basis of biological properties

On the basis of host range, reproductive cycle, cytopathology and characteristics of latent infection herpesviruses have been classified into three sub-families, referred to as alpha, beta and gammaherpesvirinae (Barahona *et al.*, 1974; Honess and Watson, 1977; Honess, 1984; Roizman *et al.,* 1981; Roizman, 1982).

Alphaherpesvirinae

Many members of this subfamily are neurotropic and exhibit a wide host range *in vitro*. Their productive cycle is short and they spread rapidly in tissue culture with mass destruction of infected cells. Latent infections are frequently established in the sensory ganglia (Wildy *et al.*, 1982). Members of this group include HSV1, HSV2, VZV, PRV, EHV1, equine herpesvirus type 3 (EHV3), equine herpesvirus type 4 (EHV4) and bovine herpesvirus type 2 (BHV2).

Betaherpesvirinae

This group exhibits a restricted host range and a long reproductive cycle. Infected cells often became enlarged (cytomegalia) and persistently infected cell cultures are readily established. The establishment of latency may occur in a variety of tissues including secretary glands, kidneys and lymphoreticular cells. HCMV is a member of this subfamily.

Gammaherpesvirinae

This sub-family also has a narrow host range which is specific either for B lymphocytes, the gamma 1 subgroup, e.g. EBV, or T lymphocytes, the gamma 2 subgroup, e.g. herpesvirus saimiri (HVS). The length of the reproductive cycle and cytopathology are variable and latent infections are frequently established in lymphoid tissue. This group includes EBV, HVS, equine herpesvirus type 2 (EHV2), equine herpesvirus type 5 (EHV5) and bovine herpesvirus type 4 (BHV4)

1.2.2. Subclassification on the basis of genome structure

Comparisons of the predicted amino acid sequences have shown that genetic relationships of individual viruses agree in general with the biological classification. However some exceptions do exist, e.g. bovine herpesvirus type4 (BHV4) has a long replicative cycle and is enveloped primarily by budding on smooth cytoplasmic membranes of Golgi body elements to give rise to the formation of cytoplamic inclusions. On this basis it was classified as a cytomegalovirus (Storz *et. al.*, 1984). However, the BHV4 genome is 144 kbp in size and has polyrepeated DNA segments at both termini. This bears closer resemblance to the genome of the lymphotropic virus HVS, a member of the gammaherpesvirinae, than to the cytomegalovirus (Ehlers *et al.*, 1985). Furthermore, the recently identified HHV6, was originally assigned to the gammaherpesvirinae on the basis of its tropism for lymphocytes. However, on the basis of its sequence homology and gene organisation, HHV6 is more related to HCMV and more properly belongs to the betaherpesvirinae than gammaherpesvirinae. EHV2 and EHV5 were previously classified as betaherpesviruses. However, sequence analysis has revealed that EHV2 and EHV5 share greater amino acid sequence similarity with EBV and HVS than other herpesviruses. On the basis of genome similarity and biological properties, they were reclassified as gammaherpesviruses.

1.3. THE PATHOGENESIS OF EQUINE HERPESVIRUSES

1.3.1. Introduction

Equidae are susceptible to infection by at least five viruses of the herpesviridae, EHV1, 2, 3, 4 and 5.

EHV1, an alphaherpesvirus, is responsible for respiratory, sporadic and epizootic abortigenic and neurologic diseases. Epizootic abortion caused by EHV1 infection can cause economically devastating losses (Plummer and Waterson **1963).**

EHV2 and EHV5 are gammaherpesviruses. EHV2 originally comprised an antigenically and genomically heterogeneous group of viruses (Browning and Studdert, 1987). Considerable antigenic and biological diversity among EHV2 isolates has been reported. It was found that the numbers of hours prior to the commencement of viral replication and the time required for completing virus induced cytopathology varied across different isolates of EHV2. A large degree of genetic heterogeneity between EHV2 isolates was also identified by restriction endonuclease analysis (Browning and Studdert, 1987). Based on these findings, the existence of a new type of equine herpesvirus was proposed, and EHV2 was subdivided into EHV2 and a new type, provisionally designated EHV5 which has growth characteristics similar to EHV2.

EHV2 and EHV5 were previously classified as betaherpesviruses based on their resemblance in many of their biological properties to CMV of man and other animals (Hsiung *et al.*, 1969; Plummer *et al.*, 1969). However, EHV2 and EHV5 share certain biological properties with viruses classified in the gammaherpesvirinae. They are not restricted to primary cell culture from natural host species. They grow not only in equine cells, but also in rabbit and feline kidney cells (Plummer *et al.*, 1969, 1973) . Contrary to the provisional classification of EHV2 and EHV5 as betaherpesviruses, it has been shown (Telford *et al.*, 1993) that EHV2 and EHV5 share greater amino acid sequence similarity with EBV and HVS than with other herpesviruses. In 1994, Agius and Studdert reclassified

EHV2 and EHV5 as gammaherpesviruses based on their genomic relationship and biological features.

The natural route of infection of EHV2 appears to be the upper respiratory tract, as suggested by the epidemiological similarities between EHV2 and EHV5 and EHV1 and EHV4 (Bagust *et al.*, 1972; Sherman *et al.*, 1977). In contrast to knowledge of the disease produced by EHV1 and EHV4, the consequences of infection of horses by EHV2 and EHV5 are largely unknown (Plummer *et al.*, 1963; Erasmus , 1970). Although the incidence of infection of horses by EHV2 and EHV5 has been shown virologically to be very high, little evidence has been forthcoming which identified the virus unequivocally as the particular or initiating cause of any disease. The viruses can be isolated from normal tissues, such as the respiratory tract, leukocytes and conjunctivas in both naturally (Plummer, *et al.*, 1963) and experimentally infected horse (Gleeson, and Studdert, 1977; Bryans and Prickett, 1969).

EHV3, a second equine alphaherpesvirus, is the cause of a benign progenital exanthematous disease (Ludwig, *et al.*, 1971; Bryans and Allen, 1973). EHV3 infection causes small seldom noticed vesicles in the skin of the vulva or the penis. These lesions may enlarge peripherally for a few days and may coalesce to form larger erosions. Secondary bacterial infections localised to the viral lesion may produce severe necrotising balanitis which is medically the most serious consequence of the infection. Progenital disease occurs in maiden colts and fillies. Infection by the virus can be acquired by the respiratory route and infection appears not to constitute a threat of abortigenic disease (Bryans and Allen, 1973).

EHV4, an alphaherpesvirus, is responsible for respiratory disease of young animals as well as for sporadic and epizootic abortigenic disease. For a long time, EHV1 and EHV4 were believed to be the same virus, named as rhinopneumonitis virus (ERV) (Doll and Bryans, 1957; Doll *et al.*, 1956). In 1959, Shimizu *et al.* found EHV1 could be divided into two subtypes on the basis of cross-neutralization tests. This was subsequently confirmed by

other workers (Matumoto *et al.*, 1965, Mayr *et al.*, 1965; Borgen and Ludwig, 1974). The epidemiological data have shown that although respiratory disease is common in all horse populations, EHV1 induced abortion has a far higher incidence in the U. S. A. and parts of Europe than it does in Australia, South Africa, Japan and Great Britain (Platt, 1973; Burrows and Goodridge, 1979; Coggins, 1979; Mumford and Rossdale, 1980). The relative rarity of EHV1 abortion in Ireland, where rhinopneumonitis occurs annually in young stock, led Farrelly to suggest, as early as 1966, that there are two strains of EHV1: an abortigenic strain and a non-abortigenic strain. Using endonuclease analysis of virus DNA, Sabine *et al.* (1981) first demonstrated that there are two subtypes of EHV1. Virus isolates within each subtype have similar restriction endonuclease profiles which are entirely different from the other subtype. Using restriction endonuclease analysis in epizootiological studies of EHV1 and EHV4, Allen *et al.*, (1983) and Studdert, (1983) found that EHV4, like EHV1, causes epizootics of respiratory disease in young horses, but is rarely the cause of abortion storms or the neurological syndrome. Kinetic analysis of DNA and DNA reassociation suggests that the EHV1 and EHV4 share only 17% of their genome nucleotide sequence (Allen and Turtinen, 1982). Based on these studies, Studdert et al., (1981) suggested that viruses with a respiratory subtype or subtype 2 restriction enzyme pattern be renamed EHV4. Both subtypes are respiratory pathogens but only viruses which have a subtype 1 (EHV1) restriction enzyme pattern are commonly associated with abortion.

1.3.2. Abortion caused by EHV1

Though abortigenic disease in an individual mare can conceivably arise as a result of recrudescence of a latent infection, most such disease results from a progression of pathological events originating from reinfection of the respiratory tract in an immunologically experienced mare during the terminal four months of pregnancy (Bryans and Prickett, 1969). Because abortigenic disease is a disease of immunologically experienced individuals, infection of such individuals usually does not result in appearance of clinically detectable respiratory disease. The incubation period, from infection of the

respiratory tract until abortion varies from 9 days to, in rare cases, several months (Doll and Bryans, 1962). If foetuses are inoculated directly with the virus, abortion always ensues within 3 to 9 days, which is the incubation period from infection of the susceptible foetus to abortion.

As opposed to most foetuses aborted as a result of microbial placentitis or infection of mares by equine arthritis virus, those infected by EHV1 show no postmortem autolytic changes. Demonstrable lesions are confined to the foetus, no gross or microscopic lesions of the viral infection have been described on the placental tissues, and the majority of foetuses are viable until immediately before they are aborted. Histologically, the lung presents lesions of bronchopneumonia with necrosis of bronchial epithelium and pathognomonic intranuclear herpetic inclusion bodies which are most prominent in epithelial cells of the small bronchi. The liver is hyperemic and may contain miliary petechial hemorrhages and areas of focal necrosis.

Although the lesions demonstrable in aborted foetuses are qualitatively the same, they vary quantitatively to a great degree. It appears however that , no matter how severe the lesions, the disease of the foetus is not incompatible with its life *in utero* during the period from infection to abortion. It has been suggested that lesions histologically demonstrable in the uteri of mares after their foetuses were infected by transplacental inoculation are interpretable as evidence that abortion is an immunologically mediated phenomenon (Bryans and Prickett, 1969).

1.3.3. Respiratory disease

Respiratory disease caused by EHV1 and EHV4 is a disease of young, immunologically inexperienced horses. The primary infection is usually contracted during their first year of life, frequently about the time of weaning. After an incubation period of about forty hours, disease presents as febrile respiratory illness. The relative severity of physical symptoms of disease appears, from observation of both naturally acquired and experimentally induced

infections of young horses, to be related to the infecting virus subtype, i.e. disease caused by EHV1 virus is generally more severe than disease caused by EHV4. The viral infection in experimentally infected foals produces an acute brochopneumonia with necrosis of the bronchial epithelium. The virus infects the upper respiratory mucosa causing vesiculation of the epithelium in which the temperature may reach 41.5 °C. Infected foals and yearlings display a serious trickling nasal efflux early in the course of disease. The lesions produced by virus in the upper respiratory tract are routinely superinfected by bacteria (Streptococcus Zooepidemicus). The secondary infection may produce pyrogenic tonsillitis and inflammatory hypertrophy or abscessation of the retropharyngeal and intermandibular lymph nodes and commonly cause bronchopneumonia especially in foals (Prickett, 1970). The morbidity rate within herds of immunologically naive weanlings approaches 100%.

1.3.4. Neurological disease associated with infection of EHV1

Neurological disease associated with infection of EHV1 was identified by Saxegaard (1966) who first isolated EHV1 from two adult horses which showed serious neurological symptoms. These symptoms caused by EHV1 can affect animals of any age, including suckling foals (Jackson and Kendrick, 1971). It may be preceded either by the occurrence of respiratory disease in young animals or abortions, or as the only clinical manifestation of infection by the virus. Infection is acquired via the respiratory tract and young horses may therefore show signs of respiratory disease. The earliest sign of neurological disease is a proprioceptive defect of the hind limbs, evident by a reluctance to move or ataxia with dragging of the feet. These signs are the result of lesions in the spinal cord resulting from vasculitis. In contrast to herpesviral encephalomyelitis in other species, neurological disease associated with infection of horses by EHV1 apparently does not occur as a result of a productive viral infection of the central nervous system (CNS). There is no evidence of infection of neurones or other cells of the CNS and virological evidence for such infection (Little and Thorsen, 1976; Charlton *et al.*, 1976) is rendered equivocal by the fact that EHV1 viraemia is demonstrable in many cases of the disease. Jackson *et al.* (1977) suggested that EHV1 produces a neurologic syndrome which results from a pathogenic

mechanism apparently unique among herpesviruses. Vasculitis in affected horses is not confined to the CNS. Similar lesions have also been demonstrated in the endometrium, uvea, nasal mucosa, lungs and at other anatomical sites (Little and Thorsen ,1976; Charlton *et al.*, 1976; Jackson *et al.*, 1977) in affected animals. Although the virus is not routinely isolatable from the CNS, it is commonly present in peripheral blood leukocytes during the course of CNS disease. Histological evidence (inclusion bodies) of viral infection of endothelial cells in the equine foetus as well as evidence for the presence of viral antigen in endothelial cells of vessels of the CNS of horses with EHV1 neurological disease has been presented (Patel et al., 1982).

Evidence for the presence of immune complexes, presumed to be EHV1 viral antigen-antibodies, in the serum of horses inoculated with a "paretic" EHV1 isolate and for thrombocytopenia indicative of the early formation of thrombi in the same experimental subjects has been presented (Jackson *et al.,* 1977). These workers demonstrated that the initiation of the inflammatory alterations of vessels in the CNS is proliferation and necrosis of endothelium. The presence of viral antigen in the endothelium and the pattern of progressive histological alterations of vessels which leads to functional damage to the CNS, suggests that EHV1 encephalomyelitis of the horse is the result of a generalised Arthus (type III) immunological reaction analogous to that which is responsible for equine purpura haemorrhagica.

1.3.5. EHV 1 latency

An ability to establish latent infections is crucial to the survival of herpesviruses in the immune environment which results from the host's reaction to infection. Alphaherpesviruses use neurological tissue as their site of latency. It has been demonstrated that after infection with HSV at a peripheral site and the invasion of the sensory nerve endings, the virus ascends by retrograde axonal transport (Oches, 1974; Kristensson, 1988) in nerve axons to the nuclei of associated sensory ganglia. At this stage,

either viral replication resulting in neuronal destruction occurs or a latent infection is established.

Periodically the latent viral genome may be reactivated spontaneously. It passes down the axon to epithelial cells at or near the primary site of infection where a productive infection occurs. Using a combination of *in vitro* tissue culture and animal models, latency may be functionally dissected into at least four sequential stages: (i) viral replication in the peripheral tissue in the absence of viral replication in the ganglia; (ii) establishment of a latent state in sensory nerves; (iii) subsequent maintenance; and (iv) reactivation of the latent virus leading to a productive infection at or near the primary site of inoculation.

It has been demonstrated that, depending on the site of primary infection, latency can be established in any peripheral nervous tissue, including the autonomic ganglia (Price *et. al,* 1975; Martin *et al.,* 1977; Warren *et al., 1*978). *In situ* hybridisation (Cook *et al.,* 1974) and immunofluorescence analysis (McLennan and Darby, 1980) have confirmed that latent virus reactivates from the neurones of ganglia. Following corneal inoculation of HSV, examination of DNA isolated from latently infected murine ganglia and central nerve system (CNS) revealed that the latent DNA was in a different physical form compared to that observed both during acute infection and in virions. Rock and Fraser (1983) demonstrated a lack of terminal fragments of HSV DNA in latently infected tissue. Efstathiou *et al.,* (1986) confirmed that latent HSV DNA in murine and human trigeminal ganglia is in the endless configuration.

Given that virtually any HSV1 deletion mutant can establish latency in mice (Weber *et al.,* 1987; Sears *et al.,* 1985; Leib *et al.,* 1989; Katz *et al.,* 1990) and that viral DNA replication (Steiner et al., 1990; Harris and Preston, 1992) and gene expression (Sederati *et al.,* 1989) are not prerequisites for latency, it is possible that cellular factors play an important role in the repression of viral gene expression. The maintenance of the latent state of HSV is dependent upon the presence of nerve growth factor (NGF). Deprivation of NGF leads to

the destabilisation of latency in primary sympathetic and sensory neurones in culture (Wilcox and Johnson, 1988; Wilcox *et al.*, 1990). In addition *in vivo*, the severing of sensory neurones prevents the transport of NGF to the neural cell body and results in reactivation (Carton and Kilbourne, 1952).

Transcription of the HSV genome during the latent phase is restricted to the latency associated transcripts (LATs), which were identified in murine, rabbit and human latently infected sensory ganglia by *in situ* hybridization (Stevens *et al.*, 1987; Rock *et al.*, 1988; Croen *et al.*, 1988; Stevens *et al.*, 1988). LATs are located in the long repeat region of the HSV1 genome and are therefore present in two copies per genome. LATs initiate 3' to the IE1 gene and are antisense to the IE1 transcripts. The primary LAT transcript is 8.3 kb which gives rise to a family of LAT RNAs, including very stable ones of 2 and 1.5 kb (Croen *et al.*, 1987; Gordon *et al.*, 1988; Rock *et al.*, 1987; Spivack and Fraser, 1987). Sequence analysis of the LATs revealed that the predominant LAT species contains two putative ORFs, ORF1 and ORF2 (Wechsler *et al.*, 1989). Despite the presence of these ORFs, no protein products from the predicted LAT ORFs have been detected (Spivack and Fraser, 1987; Wagner *et al.*, 1988).

As LAT deficient viruses are capable of establishing, maintaining and reactivating from latency, it appears that LATs are not essential at any stage of the lytic cycle (Steiner *et al.*, 1989; Javier *et al.*, 1988; Sederati *et al.*, 1989). However LAT is important for efficient reactivation from sensory neurons, since LAT transcription-negative mutants have been shown to reactivate poorly by transplant or induced reactivation in the mouse (Block *et al.*, 1990; Hill *et al.*, 1990; Ho, and Mocarski, 1989; Javier *et al.*, 1988). The role of LAT in latent infection is still unclear.

A large number of induction procedures have been developed which reactivate latent HSV *in vivo*. Trauma to the ganglia or neurectomy prevents, or substantially reduces, the transport of NGF to the neural cell body, and leads to the reactivation of latent virus (Walz

et al., 1974; Berger *et al.*, 1990; Price and Schmitz, 1978). Reactivation can also be induced by physical or chemical traumas. In addition, manipulation of the host immune system by the administration of immunosupressive drugs (Openshaw *et al.*, 1979; Hurd and Robinson, 1977), superinfection by bacteria (Stevens et al., 1975) and anaphylactic shock (Good and Campbell, 1948), all induce the reactivation of latent HSV.

Although comparatively little effort has been applied to investigation of latency for equine herpesviruses, compared to that for HSV, convincing evidence has accumulated which supports the concept that equine viruses survive as a result of sharing with other herpesviruses the ability to establish themselves latently in their natural host species. Epizootiological observations of diseases caused by EHV1 provides circumstantial evidence that stresses resulting from such influences as transport, other infections or vaccinations, mixing of horses from different herds together at sales or race meetings increase the likelihood of the occurrence of herpesviral disease (Bryans and Allen, 1989). Edington *et al.* (1985) and Browning (1988) presented the first experimental evidence that EHV1 could exist in a latent form and be reactivated following immunosuppression with dexamethazone and prednisolone. They found that after three months of initial infection, EHV1 was recovered from six ponies in a group of eight within two weeks of corticosteroid treatment. No clinical signs of respiratory disease were detected after virus reactivation. The two animals from which virus failed to be recovered, showed a significant increase in complement fixing antibody suggesting that reactivation has gone undiagnosed at both the clinical and laboratory level (Mumford, 1985). Welch *et al.* (1992) found that ten weeks following an experimental infection of five ponies with EHV1 and EHV4, latent virus DNA could be detected by PCR using primers from the gB gene. Additionally virus was predominantly recovered from the lymphoid tissues of the respiratory tract by co-cultivation. In addition, latent EHV1 and EHV4 persisted at significant levels in PBL.

Slater *et al.* (1994) demonstrated that the trigeminal ganglion is a location for EHV 1 latency and subsequent reactivation in experimentally infected horses. Four specific

pathogen-free (SPF) ponies were infected intranasally with EHV1. No virus was detected in clinical specimens by 10 days post-infection. Two months later a reactivating stimulus was administered to all ponies and virus was shed into the nasal mucus (for 10 days) which proved the presence of a latent infection. After a further 6 weeks, co-cultivation of explanted trigeminal ganglia from two out of the four ponies yielded cultures positive for infectious virus. Apart from the nasal epithelium, no infectious virus was recovered from any other tissue. PCR also confirmed the presence of virus DNA in the ganglia from six ponies. These data shows that EHV1 in common with other members of the alphaherpesvirus subfamily establishes latency in sensory ganglia from which virus can be reactivated. These observations suggested that a site of latency from which virus can reactivate is the trigeminal ganglion, which is epidemiologically important for reactivation because reactivated virus from it may be directly transferred to peripheral respiratory tissues from which transmission to susceptible hosts can occur.

Baxi *et al.* (1995) have investigated LATs of EHV1 in neural tissues obtained from SPF ponies that had been experimentally infected with EHV1. They labelled the EHV1 genomic fragments and carried out *in situ* hybridization to RNA from latently infected trigeminal ganglia. Six positive neurones were observed among approximately 3000 neurones examined (0.02%). Only one fragment , the BamHI E fragment (n.p. 108710-118620) yielded positive results in sections obtained from 2 out of 4 ponies. The BamHI E fragment contains EHV1 genes 64 (HSV1 homologue IE175), 63 (HSV1 homologue IE110) and the 5' end of gene 62 (HSV1 homologue UL1). The use of subcloned probes from this fragment showed that the region around the 3' end of gene 63 is transcribed during latency. Nothern blots with riboprobes revealed that the LATs are transcribed from the strand opposite to gene 63 and localised to the neuronal nuclei. These findings demonstrated that (i) equine neurones are a cellular site of EHV1 latency; (ii) a very small proportion of neurones contained transcriptionally active EHV1 during latency; and (iii) at least one region of the EHV1 genome is transcriptionally active during latency (Baxi *et al.*, 1995). These findings are consistent with those obtained for HSV1, PRV1 BHV1 and

feline herpesvirus type 1 (FHV1), where latency transcripts mapping antisense to immediate early genes have been demonstrated in sensory ganglia.

Sequence analysis of the EHV1 BamHI E fragment has showed that the region located approximately 700 bp from the 3' end of gene 63 has a RNA polymerase II transcription site and is similar to HSV1 LAT, including a TATA box, CAAT box, SP1-binding site and LPBF region. The putative EHV1 LPBF (CCAGCTGG) differed from the HSV1 LPBF only in the interchange of two central bases in the palindromic sequences, where GC is in the EHV1 promoter region, instead of CG (Zwaagstra *et al.,* 1991; Baxi *at al.,* 1995). Since deletion of the HSV LPBF causes a decrease in promoter activity in the transcription of HSV1 LATs (Zwaagstra *et al.,* 1991), it is speculated that the change observed in the EHV1 genome may decrease the promoter activity for EHV1 LATs; it may also explain why a very low number of LAT-positive neurones were observed. At the moment the role of the EHV1 LATs in virus latency is unknown.

1.4. IMMUNE RESPONSE TO EHV1 INFECTION IN THE HORSE

Infection of horses by EHV1 results in an immune response demonstrable by development of antibodies, a T cell response to viral antigens and development of resistance to reinfection (Bryans, 1969; Pachciarz and Bryans, 1976). The primary virus neutralizing antibody (SN) response to infection acquired by intranasal administration of virus is detectable eight to nine days later. Complement fixation antibody titres reach their highest level about three weeks after primary infection but are no longer detectable after 60 days. The SN antibody responses are more persistent and last for more than a year (Burrows and Goodridge, 1973). In addition to the development of specifically reactive immunoglobulins which are detectable in both serum and nasal secretions (Thompson, 1978), the occurrence of T cell responses to EHV1 infection can be demonstrated by viral antigen induced transformation of PBL (Pachciarz and Bryans, 1976) and development of delayed type hypersensitivity and immune lymphocyte cytotoxicity for virus infected cells (Bryans,1969)

in naturally or experimentally infected horses. Resistance to reinfection of the respiratory tract is of short duration and immunologically experienced horses may be reinfected repeatedly. Such reinfection may be accompanied by cell-associated viraemia and may result in abortion or neurological disease (Bryans, 1969). The virological and immunological (antibody response) measurements illustrate that immunity to reinfection of horses by the same virus which produced a previous infection lasts for as short a period of time as 3 months.

1.5. STRUCTURE OF THE EHV1 VIRION

The EHV1 virion is morphologically indistinguishable from other members of the herpesvirinae. The virion is approximately 150-170 nm in size and is composed of four major subvirion components: the envelope, tegument, capsid and core (Plummer and Waterson, 1963; Abodeely *et al.,* 1970; Darlingon and Moss, 1968; O'Callaghan and Randall, 1976).

Abodeely *et al.* (1970) found, using comparative electron microscopy, that EHV1 capsids are approximately 100 nm in diameter and contain a linear double stranded DNA molecule. The morphologically distinctive herpesvirus capsid contains 162 capsomers. 12 capsomers are 'pentons' and 150 capsomers are 'hexons' which show 5:3:1 axial symmetry (Wildy *et al.,* 1960; McCombs *et al.*, 1971). Depending on their positions on the surface lattice the hexons are classified into three P, E and C (Stevens *et al.,* 1986). Perdue *et al.* (1975) found that three distinct species of EHV1 nucleocapsids were isolated from infected mouse fibroblast (L-M) cell nuclei which were classified on the basis of their densities in Renografin gradients as Light (L), Intermediate (I) and Heavy (H). These capsids comprised six major proteins (I, II, III, IVa and V) and several minor proteins, each comprising less than 1% of the total nucleocapsid protein. Analysis of the DNA content of the three nucleocapsid species indicated that preparations of H nucleocapsids contain more DNA than do those of the I and L nucleocapsids species. L and I nucleocapsids lack a dense inner core structure characteristic of the H species (Perdue *et al.*, 1975). Using

biochemical assays, Newcomb *et al.*,(1989) demonstrated that neither L nor I nucleocapsids contained any significant amounts of DNA.

The capsid is surrounded by an amorphous asymmetrical material designated the tegument (Roizman & Furlong, 1974). Several EHV1 proteins have been tentatively assigned to this structure (O'Callaghan *et al.*, 1983; Turtinen, 1983; Allen and Bryans, 1986). The final outermost component is the viral membrane or envelope, which is probably derived from the host cell nuclear membranes (O'Callaghan and Randall, 1976; Darlington and Moss, 1968). In herpesviruses, this triple layered structure includes viral glycoprotein spikes projecting from the surface (Manservigi*et al.*, 1977; Sarmiento *et al.*, 1979; Noble *et al.*, 1983; Buckmaster *et al.*, 1984; Spear, 1993a; 1993b). Six major and six minor glycoproteins have been identified in the envelope of EHV1 (Turtinen and Allen, 1982; Turtinen, 1983; Allen and Bryans, 1986). The six major glycoproteins are between 240,000 and 40,000 in molecular weight, and are designated gp2, 10, 13, 14, 17/18 and 22a based on the original nomenclature of O'Callaghan and Randall, (1976). Furthermore, gp13, gp14, and gp17/18 mapped to positions collinear with HSV glycoproteins B, C, and D (Allen and Yeargan, 1987; Allen and Coogle, 1988; Whittaker *et al.*, 1992b). gp2 (gp300 or gene 71 protein) has been characterised as an O-linked class I membrane glycoprotein (Whittaker *et al.*, 1990, 1992a) encoded by EHV1 gene 71 (Sun *et al.*, 1994) which is non-essential for virus growth *in vitro.* (Sun and Brown, 1994). Sequence analysis has predicted that EHV1 encodes at least 12 membrane-associated glycoproteins which are counterparts of HSV1 glycoproteins gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, gN and UL20 protein (Telford *et al.*, 1992). Alphaherpesvirus membrane proteins play an important role in virus entry into cells, egress from cells and transmission from cell to cell (Manservigi *et al.*, 1977; Sarmiento *et al.*, 1979; Noble *et al.*, 1983; Buckmaster, 1984; Spear 1993a, b). They also play important roles in interaction with the host immune system and in determination of viral pathogenicity (Powell ., 1979; Bishop *et al.*, 1983; 1984).

1.6. THE EHV1 GENOME

The EHV1 genome is a large, linear double-stranded DNA molecule. It contains 150,223 bp and the G+C content is 56.7% (Telford *et al.*, 1992). Analysis of the complete DNA sequence, electron microscopy and restriction endonuclease mapping have demonstrated that EHV1 consists of two covalently linked components, referred to as the long (L) and short (S) segments (Whalley *et al.*, 1981; Henry *et al.*, 1981; Ruyechan *et al.*, 1982; Telford et al., 1992). The S component comprises an unique sequence (U_S) flanked by a large inverted repeat (IR_S/TR_S). The L component consists of an unique sequence (U_L) flanked by a small inverted repeat (IR_L/TR_L). The sizes of these components are: U_S, 11,861 bp; TR_S/IR_S, 12,714 bp; U_L, 112,870 bp; IR_L/TR_L,32 bp. The S region inverts leading to the presence of two equal molecular isomers in EHV1 virion DNA which differ in the orientations of U_S as indicated by the existence in certain restriction endonuclease digestions of three terminal fragments (2 0.5M and 1 1M) and two 0.5 M joint fragments (Henry *et al.*, 1981; Whalley *et al.*, 1981). Based on the arrangement of reiterated sequences of genomes, herpesviridiae fall into 5 classes (A to E). The viral DNA of group D is characterized by having two unique regions (U_L and U_S) with U_S being flanked by inverted repeats (IR_S and TR_S) which allow inversion of Us, giving rise to 2 isomeric forms. EHV1 belongs to this group (Figure 1) (Chowdhury *et al.*, 1990; Yalamanchili and O'Callaghan, 1990).

DNA sequence analysis identified a high percentage of the genome as having protein coding potential which is tightly packed and is present almost equally on both DNA strands. A total of 83 open reading frames (ORFs) with the potential to encode 76 distinct polypeptides were initially identified within the genome (Figure 2). Sixty three genes, 1-63 are located within the U_L region and nine genes (68-76) within U_S. Genes 64-67 are in the IR_S/TR_S repeat sequences and therefore are represented twice (Telford *et al.*, 1992). A feature of the EHV1 genome is that several transcription units overlap and share a common polyadenylation site with transcribed mRNAs of different lengths. A similar feature is also found in the HSV1 genome (McGeoch *et al.*, 1985). ORFs 44 and 47 are homologous to

Figure 1. EHV1 genome arrangement and isomers.

The horizontal lines represent EHV1 unique sequences (U_L and U_S) and filled rectangles represent EHV1 repeat sequences larger than 1Kbp in length (IR_S and TR_S). The arrows indicated the relative orientation of L and S segments in the isomers. (a) indicates repeat sequence with (a') its complement.

EHV1 GENOME

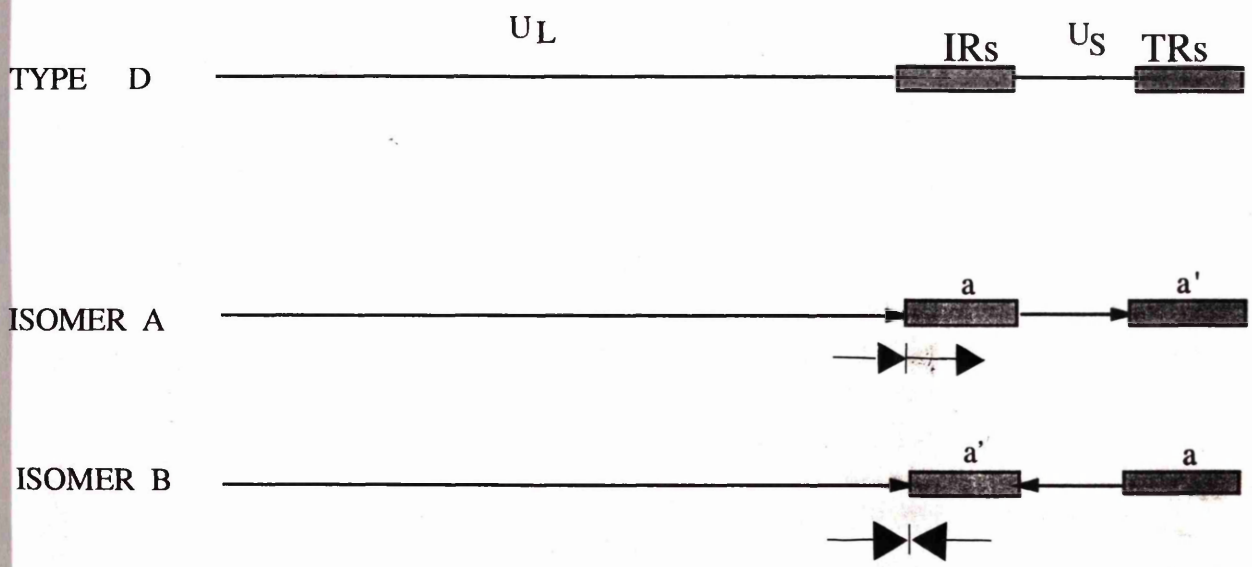
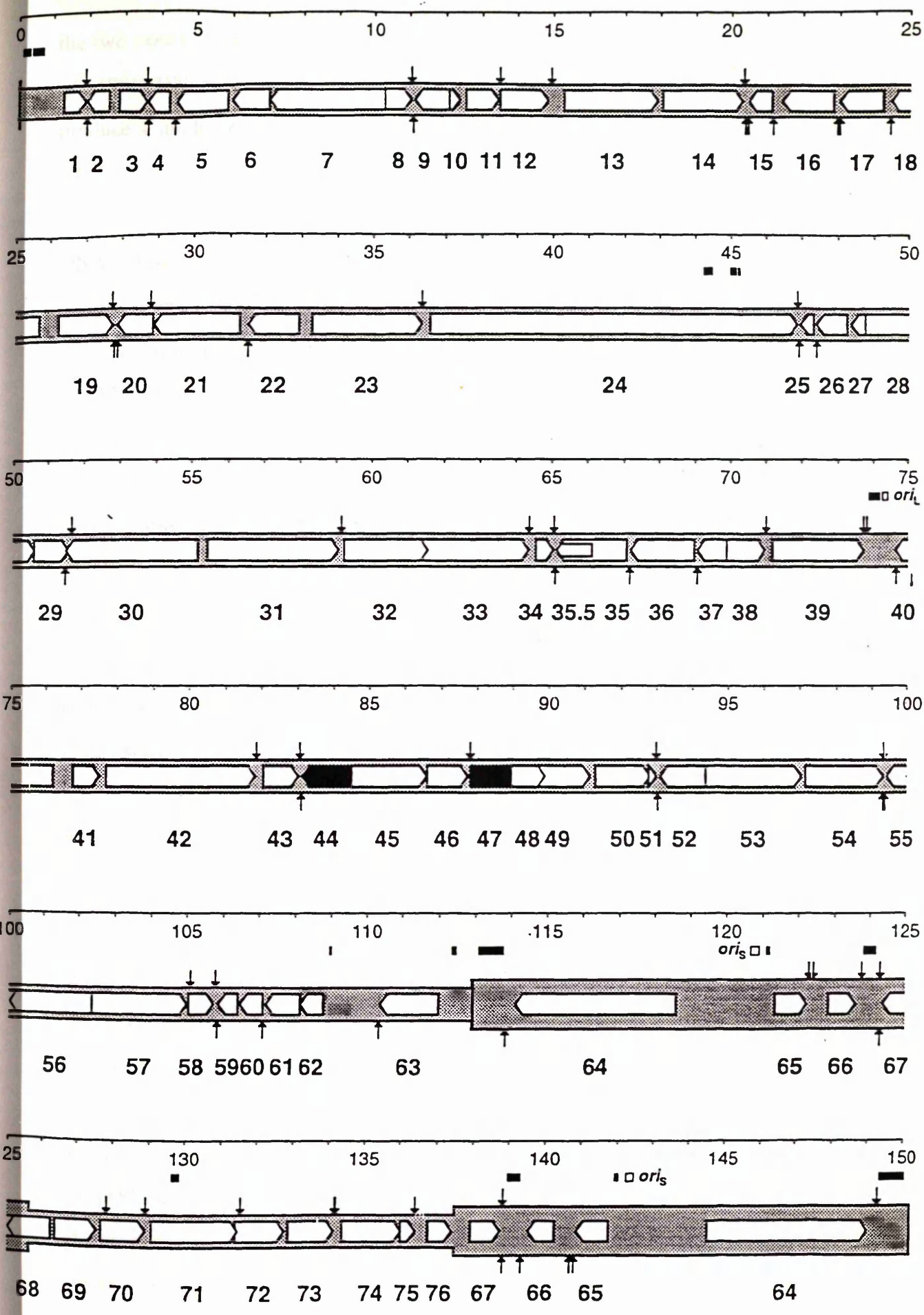


Figure 2. Predicted EHV1 gene ORFs and gene arrangement.

The genome is shaded, the thinner and thicker portions denoting the unique regions (U_L and U_S) and inverted repeats (TR_L, IR_L, TR_S, IR_S), respectively. The scale is in kbp. Protein-coding regions are shown as open arrows with gene nomenclature below. ORF 35.5 is shown by a thinner arrow to reveal the region of overlap with ORF 35. ORFs 44 and 47, represented as filled arrows, are probably expressed as a spliced mRNA. Vertical arrows indicate candidate polyadenylation sites in the appropriate strand. The locations of reiterations (■) and candidate origins of DNA replication (□) are indicated above the genome.

It is published by Telford et al., 1992.


0
5
10
15
20
25
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
25
30
35
40
45
50
19 20 21 22 23 24 25 26 27 28
50
55
60
65
70
75
ori
29 30 31 32 33 34 35.5 35 36 37 38 39 40
75
80
85
90
95
100
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55
100
105
110
115
120
125
ori
56 57 58 59 60 61 62 63 64 65 66 67
125
130
135
140
145
150
ori
68 69 70 71 72 73 74 75 76 67 66 65 64

the two exons of the HSV1 gene UL15, which is expressed as a spliced mRNA (Costa *et al.*, 1985; Dolan *et al.*, 1991), and are probably also expressed as a spliced mRNA to produce a single protein. A second ORF, gene 35.5 has been mapped within the gene 35 ORF.

DNA hybridisation studies and DNA sequence analysis indicated that the EHV1 genes are closely colinear with their counterparts in HSV1 and VZV. The gene layout of EHV1 is very similar to that of HSV1 and VZV although UL is in the inverted orientation compared to HSV1 (Telford et al. 1992; Davison and Wilkie, 1983a). Only three EHV1 genes (genes 1, 67 and 75) have no position and sequence counterparts in VZV and HSV1, while another five EHV1 genes (genes 2, 15, 59, 70, 71) lack significant sequence homology in VZV and HSV1 (Telford *et al.*, 1992).

Most of the predicted EHV1 gene products remain unidentified and their functions are not known. However, based on the complete DNA sequence data and present knowledge of HSV1, the properties or functions of EHV1 proteins are predicted in table 1 (Telford *et al.*, 1992 ; Whalley *et al.*, 1989; Allen and Coogle, 1988; Guo *et al.*, 1989; Flowers *et al.*, 1991; Whalley *et al.*, 1991; Audonnet *et al.*, 1990; Elton *et al.*, 1991; Tutinen and Allen, 1982; Whittaker, *et al.*, 1992a,b; McGowan *et al.*, 1994; Jöns *et al.*, 1996). Among the known or putative EHV1 proteins, eight class I membrane proteins are encoded by EHV1 genes 33, 16, 72, 74, 39, 73, 6 and 10, which have high homology with their counterparts (gB, gC, gD, gE, gG, gH, gI, gK and gN) in HSV1. These glycoproteins share three features in common. First, they are all predicted to have cleavable N-terminal signal sequences which include a particularly hydrophobic amino acid region of around 10 residues and one or more residues with charged side chains between the N-terminus and the hydrophobic region. Second, these proteins all have hydrophobic regions near their C-terminus which are predicted to serve as membrane spanning domains. Third, these proteins are all glycoproteins and have potential sites for the addition of N-linked oligosaccharides (Spear, 1984). One additional EHV1 gene 71 was also predicted to encode a class I membrane

TABLE 1

CHARACTERISTICS OF EHV-1 PROTEINS

Gene	Start[a]	Stop[b]	Codons	M_r	VZV counterpart[c]	HSV-1 counterpart[c]	Properties or functions of HSV-1 proteins[d]
1	1,298	1,906	202	21,671	—	—	
2	2,562	1,945	205	23,398	1(26)	—	
3	2,941	3,614	257	28,021	2(163)	—	
4	4,249	3,647	200	22,379	3(263)	UL55(181)	
5	5,874	4,462	470	51,318	4(436)	UL54(677)	Transcriptional activator
6	7,042	6,011	343	38,042	5(666)	UL53(544)	Membrane glycoprotein
7	10,301	7,056	1081	118,956	6(1921)	UL52(1912)	DNA helicase/primase complex
8	10,300	11,037	245	26,364	7(453)	UL51(369)	
9	12,115	11,135	326	35,207	8(292)	UL50(299)	Deoxyuridine triphosphatase
10	12,084	12,386	100	10,801	9A(113)	UL49A[38]	Possible transmembrane protein
11	12,549	13,463	304	33,239	9(257)	UL49(281)	Tegument protein
12	13,505	14,944	479	53,644	10(838)	UL48(531)	Tegument protein
13	15,317	17,932	871	96,966	11(397)	UL47(280)	Tegument protein
14	18,083	20,326	747	82,305	12(987)	UL46(502)	
15	21,146	20,487	219	23,798	—	UL45[46]	Virion protein
16	22,951	21,445	468	50,887	14(365)	UL44(108)	Membrane glycoprotein (gC)
17	24,234	23,029	401	43,203	15(153)	UL43[45]	Multiply hydrophobic protein
18	25,696	24,479	405	45,084	16(185)	UL42(101)	DNA polymerase processivity factor
19	26,262	27,755	497	56,540	17(876)	UL41(706)	Host shutoff virion protein
20	28,959	27,994	321	36,015	18(1053)	UL40(899)	Ribonucleotide reductase (RR2)
21	31,276	28,904	790	88,394	19(1992)	UL39(1600)	Ribonucleotide reductase (RR1)
22	32,916	31,519	465	51,304	20(809)	UL38(762)	Capsid protein
23	33,292	36,354	1020	111,605	21(1314)	UL37(1733)	
24	36,588	46,853	3421	367,061	22(2243)	UL36(2225)	Tegument protein
25	47,311	46,952	119	13,596	23(114)	UL35[72]	Capsid protein
26	48,230	47,403	275	30,679	24(620)	UL34(565)	Possible virion protein
27	48,857	48,369	162	17,993	25(231)	UL33(254)	
28	48,763	50,625	620	67,297	26(1499)	UL32(1393)	Probable virion protein
29	50,618	51,598	326	36,524	27(198)	UL31(852)	
30	55,184	51,522	1220	135,949	28(3130)	UL30(3432)	DNA polymerase
31	55,453	59,082	1209	129,976	29(3283)	UL29(2852)	ssDNA binding protein
32	59,243	61,570	775	85,308	30(1927)	UL28(1804)	Probable virion protein
33	61,432	64,374	980	109,300	31(1980)	UL27(2020)	Membrane glycoprotein (gB)
34	64,578	65,060	160	17,305	32(158)	—	
35	67,093	65,153	646	68,576	33(785)	UL26(790)	Protease
35.5	66,142	65,153	329	34,679	33.5(86)	UL26.5(215)	Capsid assembly protein
36	68,975	67,212	587	63,689	34(1138)	UL25(1255)	Virion protein
37	69,897	69,079	272	29,186	35(438)	UL24(380)	
38	69,910	70,968	352	38,748	36(604)	UL23(590)	Thymidine kinase
39	71,192	73,738	848	92,937	37(889)	UL22(454)	Membrane glycoprotein (gH)
40	76,224	74,632	530	57,912	38(777)	UL21(482)	
41	76,793	77,512	239	26,534	39(334)	UL20(133)	Multiply hydrophobic protein
42	77,703	81,833	1376	152,175	40(3935)	UL19(4074)	Major capsid protein
43	82,083	83,027	314	33,840	41(956)	UL18(724)	Capsid protein
44	84,319	83,148	734	81,074	45/42(2222)	UL15(2016)	Possible DNA packaging protein
47	88,917	87,885					
45	84,480	86,600	706	76,316	43(1030)	UL17(1005)	
46	86,620	87,732	370	40,800	44(739)	UL16(491)	
48	88,947	89,900	317	35,814	46(310)	UL14(211)	
49	89,369	91,153	594	65,244	47(787)	UL13(638)	Virion protein kinase
50	91,305	92,831	508	56,064	48(735)	UL12(793)	Deoxyribonuclease
51	92,783	93,007	74	8,408	49(47)	UL11[43]	Myristylated virion protein
52	94,471	93,119	450	49218	50(607)	UL10(493)	Multiply hydrophobic protein
53	94,389	97,052	887	97,270	51(1933)	UL9(1587)	On-binding protein
54	97,173	99,323	716	77,769	52(898)	UL8(532)	DNA helicase/primase complex
55	100,331	99,420	303	33,852	53(586)	UL7(376)	

TABLE 1 —*Continued*

Gene	Start[a]	Stop[b]	Codons	M_r	VZV counterpart[c]	HSV-1 counterpart[c]	Properties or functions of HSV-1 proteins[d]
56	102,390	100,129	753	83,988	54(1665)	UL6(1065)	Possible virion protein
57	102,374	105,019	881	99,448	55(2877)	UL5(2321)	DNA helicase/primase complex
58	105,069	105,746	225	24,286	56(586)	UL4(376)	
59	106,415	105,876	179	19,451	57[24]	—	
60	107,115	106,477	212	23,681	58(532)	UL3(432)	
61	108,143	107,205	312	34,776	59(755)	UL2(757)	Uracil-DNA glycosylase
62	108,802	108,146	218	24,424	60(151)	UL1(109)	Membrane glycoprotein (gL)
63	111,984	110,386	532	58,627	61(259)	IE110(140)	Transcriptional activator
64	118,590	114,127	1487	154,859	62(1687)	IE175(1486)	Transcriptional activator
65	121,367	122,248	293	32,115	63(323)	US1(103)	*In vitro* host-range factor
66	122,861	123,571	236	25,032	64(146)	US10(129)	Virion protein
67	125,193	124,375	272	30,101	—	—	
68	126,274	125,018	418	46,786	—	US2(301)	
69	126,410	127,558	382	42,541	66(522)	US3(418)	Protein kinase
70	127,680	128,915	411	45,267	—	US4[25]	Membrane glycoprotein (gG)
71	129,096	131,489	797	80,338	—	US5[24]	Membrane glycoprotein
72	131,432	132,790	452	51,097	—	US6[61]	Membrane glycoprotein (gD)
73	132,898	134,172	424	46,390	67(228)	US7(177)	Membrane glycoprotein (gI)
74	134,405	136,057	550	61,180	68(220)	US8(196)	Membrane glycoprotein (gE)
75	136,054	136,446	130	14,854	—	—	
76	136,782	137,441	219	22,417	65[74]	US9(110)	Tegument protein

[a] Location of first base or its complement in first ATG, except for ORF 44, where first base in exon is given.

[b] Location of third base or its complement in stop codon, except for ORF 47, where last base in exon is given.

[c] Positional counterparts are listed regardless of the degree of amino acid sequence conservation. Genes lacking positional counterparts are indicated by hyphens. FastA scores greater than 100 are shown in parentheses and those less than 100 are shown in square brackets.

[d] Gene functions are derived from McGeoch (1989) and Davison (1991b), with additional data for the following HSV-1 genes: UL53, Debroy *et al.* (1985), Ramaswamy and Holland (1992); UL49, Elliott and Meredith (1992); UL47, McLean *et al.* (1990); UL45, H. Marsden (personal communication); UL42, Gottlieb *et al.* (1990); UL35, M. Davison, A. Davison, and F. Rixon, unpublished data; UL26, Liu and Roizman (1991b), Preston *et al.* (1992); UL26.5, Rixon *et al.* (1988), Liu and Roizman (1991a); UL15, Davison (1992); UL13, Cunningham *et al.* (1992); UL1, Hutchinson *et al.* (1992).

protein. Gene 71 is related to HSV1 US5 in location (McGeoch *et al.*, 1985b), but there is no amino acid similarity between their protein products. This gene is predicted to encode a polypeptide which possesses a single asparagine-linked glycosylation site and hydrophobic amino-and carboxy-terminal domains. It also contains an extensive region encoding a high proportion of serine and threonine residues. The region between residues 22 and 465 is composed of 87% serine and threonine residues and likely to be heavily O-glycosylated (Telford *et al.*, 1992). EHV1 genes 17, 41 and 52 are predicted to encode respectively, three multiple hydrophobic membrane glycoproteins, counterparts of HSV1 genes UL43, the UL20 and UL10 (Telford *et al.*, 1992). gM encoded by EHV1 gene 52 has been identified as a 58 KD protein, present both in infected cells and virions (Pilling *et al*, 1994). EHV1 gene 62 is also predicted to encode a glycoprotein (Telford *et al.*, 1992), the counterpart of HSV1 gL (Hutchinson *et al.*, 1992a).

A number of EHV1 glycoproteins which include the counterparts of HSV1 gB, gH, gL, gI and gE may form functionally multimeric assemblies. It has been found that the HSV1 gB counterpart forms homodimers or possibly homotrimers on virion and cell membranes (Claesson-Welsh and Spear, 1986). EHV1 gB has also been demonstrated to form a dimer in infected cells (Sullivan *et al.*, 1989); HSV1 gH and gL form a functional heteromultimeric unit (Hutchinson *et al.*, 1992a; Roop *et al.*, 1993) and coexpression of gL is essential for appearance of the processed form of gH on the cytoplasmic membrane (Hutchinson *et al.*, 1992a). A similar interaction has also been found for EHV1 gL and gH (Stokes *et al.*, 1996; Sun *et al.*,unpublished data) as well as for HCMV, HHV6, EBV and PRV (Kaye *et al.*, 1992; Liu *et al.*, 1993; Yaswen *et al.*, 1993; Klupp *et al.*, 1994). HSV1 gC, gI and gE are believed to modulate the immune response to infection; gC acts as a C3b receptor (Eisenberg *et al.*, 1987; Friedman *et al.*, 1984; McNearney *et al.*, 1987); gE and gI form a complex to function together as a Fc receptor, gE binds to the Fc region of IgG and this Fc-binding activity can be modulated by the presence of gI (Baucke and Spear, 1979; Frank and Friedman, 1989; Johnson *et al.*, 1988b). EHV1 genes 16, 73 and 74 also encode

gC, gI and gE homologues of HSV1, respectively, but whether EHV1 gC, gI and gE have the same functions as their HSV1 counterparts is not known.
EHV1 gC was also reported as a complement receptor (Huemer et al 1995).

1.7. THE EHV1 LYTIC CYCLE

The replication cycle of EHV1 has been studied in L-M cells.

In the hamster model EHV1 causes a consistently reproducible lethal hepatitis and virus is released into the blood in large amounts. All animals die approximately 72 hours post inoculation with 10^6 pfu/animal, and more than 95% of the hepatic parenchyma cells show typical intranuclear inclusions (Bracken and Randell, 1957; Gentry *et al.*, 1960; O'Callaghan *et al.*, 1972). In the mouse model, by 2 days after intranasal inoculation, the mice begin to show abnormal signs and by day 3 all mice are hunched with ruffled fur and a conspicuous weight reduction. Some mice showed signs suggesting a mild neurological involvement. The mice die from the fourth to the seventh day after inoculation. Approximately 50% of animals recover from the infection and become clinically normal by day 10. Virus is consistently isolated from the nasal tissues, trachea and lungs from all mice tested during the first week after inoculation and occasionally isolated from the CNS, eye and liver. Virus titres reach a peak at 3 to 5 days after inoculation, coincident with the time of maximum clinical signs. The virus in lungs and turbinate bones is cleared by day 12 after inoculation (Awan *et al.* 1990). The maximum virus titre was obtained from EHV1 infected monolayers of L-M cells at 18-24 hours post infection and 95% of the infectious virus is released from infected L-M cells (O'Callaghan *et al.*, 1978, 1983).

1.7.1. Initial steps in EHV1 infection

Virus infections are initiated through specific interactions between viral attachment proteins and their receptors on the surfaces of permissive cells. Entry of herpesviruses into cells appears to be a complex process involving numerous viral components, principally envelope glycoproteins, and more than a single cell surface receptor. For alphaherpesviruses the initial interaction between viral attachment proteins and cellular receptors is followed by virus penetration through the host membrane, involving membrane

fusion which causes the viral nucleocapsid and tegument proteins to be delivered into the cytoplasm. These two steps constitute the virus entry process.

It is now known that for most cell types, initial attachment of alphaherpesviruses to permissive cells is mediated by an interaction of a member of the gC family with cellular glycosaminoglycans, predominately heparin sulphate (HS) proteoglycans (Spear *et al.*, 1992; WuDunn and Spear, 1989). WuDunn and Spear (1989) demonstrated that the initial interaction between HSV and the cell membrane is binding to the cell surface receptors (HS moieties). Agents which block this interaction or enzymatic digestion of HS effectively prevent viral adsorption and subsequent penetration. Virions devoid of gC are impaired in the ability to bind to cells, but nevertheless infect cells, albeit inefficiently (Cai 1988; Herold *et al.*, 1991). It seems likely, therefore, that there is another heparin binding glycoprotein to mediate the binding of the mutant to cells. Early studies using antibodies (Dubuisson *et al.*, 1992; Fuller and Spear, 1985; Johnson *et al.*, 1990), cell membrane fractionation (Kuhn, *et al.*, 1990), and virosomes (Johnson *et al.*, 1984) suggested that gB and gD molecules are also involved in the virus attachment process. The HSV1 glycoprotein gB has been found to bind to HS under physiological conditions (Herold *et al.*, 1991). gB of pseudorabies virus (PrV) can also bind heparin coated beads; however, it does so only in conjunction with gC (Mettenleiter *et al.*, 1990; Sawitzky *et al.*, 1991). HSV1 gD has been shown to bind to a limited number of specific cellular receptors (Johnson *et al.*, 1990; Johnson and Ligas, 1988a). Brunetti *et al.*, (1994) have shown that HSV gD binds to mannose-6-phosphate receptors, although the relevance of the gD-mannose-6-phosphate receptor interaction in virus infection has yet to be established. That gD acts as a receptor-binding protein is further supported by observations that cells expressing gD are resistant to infection with HSV (Campadelli-Fiume *et al.*, 1988; Johnson, and Spear, 1989). Liang, et al. (1991) showed that affinity-purified bovine herpesvirus 1 (BHV-1) gB, gC and gD could inhibit virus attachment at 4°C and that gC inhibited not only wild-type (wt) BHV-1 but also a gC-negative mutant to a lower degree. Glycoproteins gB and gD inhibited wt and gC negative viruses to the same extent, which suggested that BHV-1

attachment is a complex event in that the wt virus uses gC to carry out the initial interaction with cellular receptors, which is followed by interactions of gB and/or gD with their receptors. Karger and Mettenleiter (1993) showed that the attachment of PRV and BHV-1 to permissive cells can be divided into two stages, an initial heparin inhibition-sensitive stage and a subsequent heparin-resistant stage. By using isogenic viruses with individual viral proteins deleted, they found that although the wt virus and the gD-negative mutant have similar initial binding, the gD-negative mutant was considerably impaired in the heparin-resistant binding, suggesting that the initial attachment of PRV and BHV-1 to cells is via a gC-HS interaction, followed by a gD-mediated HS-independent attachment. A similar attachment mechanism has also been revealed for HSV (Fuller and Lee, 1992; McClain and Fuller, 1994). Overall the available data collectively suggest that attachment of alphaherpesviruses to most permissive cells is a complex event, involving at least gC and gB binding to cellular HS and gD binding to other, undefined cellular receptors.

After the adsorption of virus onto cells, the following step is virus penetration into cells. Herpesviruses penetration can be mediated by both the fusion of the viral envelope with the cell membrane (Morgan *et al.,* 1968) and by phagocytosis whereby virions are engulfed at the surface and transported to the interior of the cells within a phagocytic vacuole (Hummeler *et al.,* 1969; Dales and Silverberg, 1969). However, the predominant pathway for productive entry of HSV1 is by fusion at the cell membrane (Rosenthal *et al.*, 1989). An electron microscopy study revealed that EHV1 particles can enter cells by a viropexis mechanism in which particles are engaged by pseudopodia, forming fusion vacuoles containing from one to numerous viral particles (Abodeely *et al.*, 1970). It has been found that the penetration of HSV KOS is 100 fold less likely to occur at a mildly acidic condition (pH 6.3) than at a physiological pH (pH 7.4) (Rosenthal *et al,* 1989). The relevant observations from electron microscopy studies have demonstrated that fusion predominates at pH 7.4 and endocytosis at pH 6.3.

It is proposed that multiple interactions involving virion glycoproteins and cell surface components are required to trigger fusion of the virion envelope with the cell plasma membrane, following the initial binding of HSV1 to the cell surface (Spear, 1993 a). A receptor-antireceptor interaction may be required. At least three HSV1 envelope glycoproteins (gB, gD and gH) have been found to play essential roles in viral penetration (Cai *et al.*, 1988; Desai *et al.*, 1988; Ligas *et al.*, 1988; Little *et al.*, 1981). It has been demonstrated that neutralising monoclonal antibodies against gB, gD and gH individually block viral penetration, but have a negligible effect on viral adsorption (Fuller and Spear, 1987; Highlander *et al.*, 1987, 1988). The most compelling evidence for this conclusion comes from the characterisation of viral mutants with deletions of the relevant genes. For HSV1 and PRV, it is clear that gB (gII), gD (gp50) and gH are each required for viral penetration, but not for the initial binding of virus to the cell surface (Cai, *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.* 1992; Rauh and Mettenleiter, 1991a; Rauh *et al.* 1991a,b; Peeters, 1992a,b). The evidence is that mutant virions devoid of each of the glycoproteins mentioned above are able to bind to cells at normal efficiency, but fail to penetrate. Polyethylene glycol, a membrane fusogen, can partially overcome the block to initiate infection.

Most members of the gB, gD and gH families encoded by herpesviruses are required for virus-induced cell fusion as well as for virion penetration (virion-cell fusion) (Cai *et al.* 1988; Ligas and Johnson, 1988; Rauh and Mettenleiter, 1991b; Peeters, 1992a, b; Wilson *et al.*, 1994; Roop *et al.*, 1993). In addition, monoclonal antibodies specific for gB, gD and gH can inhibit HSV-induced cell fusion (Navarro *et al.*, 1992; Noble *et al.*, 1983; Minson *et al.*, 1986). However, there is at least one exception, in that PRV gp50 (gD) is not required for cell fusion (Rauh and Mettenleiter, 1991b; Peeters *et al.*, 1992b). This finding and others summarized by Spear (1993a) indicated that, although cell fusion and viral penetration both require virus-induced membrane fusion, these two processes are mediated and regulated somewhat differently.

It is likely that members of other viral glycoprotein families are also required for HSV1 penetration. Two of the principal HSV 1 candidates are gK and gL. gK is a membrane glycoprotein with potential membrane-spanning domains (Debroy *et al.*, 1985; Pogue-Geile and Spear, 1987; Ramaswamy and Holland, 1992). It plays a role in controlling virus-induced cell fusion (Debroy *et al.*, 1985; Pogue-Geile and Spear, 1987) and is an essential gene. gL was found to be essential for HSV replication *in vitro* (Hutchinson *et al.*, 1992a, 1992b) due to its requirement for appropriate processing and transport of gH. EHV1 gL also forms a complex with gH (Stokes *et al.*, 1996; Sun *et al.*, unpublished data). However, whether EHV1 gL and gH also play the same role as their counterparts in HSV1 is unknown. Osterrieder *et al.* (1996b) have found that a EHV1 gene 52, gM, deletion mutant exhibits slower penetration kinetics compared with those of the parental EHV1 indicating that EHV1 gM is involved in virus penetration.

It is possible that some tegument proteins may also participate in the molecular interactions required for viral penetration. A temperature-sensitive mutant of HSV1 maping to UL25 which encodes a tegument protein (Addison *et al.*, 1984; Preston, 1990) was found to be impaired in penetration at the non-permissive temperature.

The precise roles of gB, gD and gH in viral penetration are not known. It is proposed that interactions of these proteins with other virion proteins and with cell surface components leads to fusion between the virion envelope and the cell plasma membrane. Members of the gB and gH families have been detected in all herpesviruses analyzed to date, with the conservation of amino acid sequence being considerably greater for gB than for gH. It has been shown that expression of BHV 1 gI (gB) can complement the defect of a gII (gB^-) PRV mutant, but not the other way round (Rauh *et al.*, 1991b) and coexpression of BHV1 gB allows infectivity of a recombinant HSV that has been exposed to neutralizing anti-gB antibody (Misra and Blewett, 1991). This apparent conservation of the structure and function of members of the gB family is consistent with required interactions with conserved domains of other virion proteins or highly conserved cell surface receptors.

It has been found that cells transformed to express a member of the gD family are resistant to infection by the homologous virus and, in most instances can be infected by a heterologous herpesvirus. Interference with viral infection is a property of all members of the gD family encoded by HSV 1, HSV2, PRV and BHV1 (Campadelli-Fiume *et al.,* 1988; Petrovskis *et al.,* 1988; Johnson and Spear, 1989; Chase, *et al.,* 1990; Tikoo *et al,* 1990). For HSV, it has been established that resistance to infection is due to inability of virus to penetrate into the gD-expressing cells, not to failure of virus to bind to the cells (Campadelli-Fiume *et al.,* 1988; Johnson and Spear, 1989). An understanding of the mechanism of interference could shed some light on the role of the members of gD family in penetration. There are some models to explain the mechanism. One is competition between the cell-associated glycoprotein and the virion-associated glycoprotein for a cell receptor needed for viral penetration (Campadelli-Fiume *et al.,* 1988; Johnson and Spear, 1989). Other models for gD-mediated interference suggest that cell-associated gD itself or some unidentified virion component inhibit penetration. With attention focused on the genetic determinants of viral sensitivity to interference, it has been shown that the structure of gD present in the virion is probably the principal determinant. Amino acid substitutions at position (25 or 27) of the mature form of gD can render the virus resistant to gD-mediated interference (Campadelli-Fiume *et al.,* 1990; Dean *et al*., 1994). In addition a mutant form of gD with a substitution at position 25 has been shown to lack interference activity (Campadelli-Fiume *et al.*, 1990). Mutant virions carrying gD with substitution at position 27 have been shown to exhibit altered specific infectivity in comparison with wt virus, even on cells that do not express gD (Dean *et al.,* 1994), suggesting cell factors also play a role in governing interference. HSV 1 mutants that are totally resistant to interference on Hep-2 cells expressing HSV 1 gD are only partially resistant to interference on L cells expressing HSV 1 gD (Dean *et al.*, 1994). Thus, it appears that determinants of interference include cell-associated and virion-associated gD (gp50, gIV), other virion proteins and cell components.

1.7.2. Alteration of host macromolecular synthesis

The sequential expression of herpesvirus genes during lytic infection usually occurs against a declining background of host macromolecule metabolism (Roizman and Roane, 1964; Rakusanova *et al.,* 1971). This inhibition of cellular DNA, RNA and protein synthesis, or "host shut-off" is a complex multistage process. The mechanisms involved are poorly understood. Most information about the process are from studies on HSV1. Host shut-off induced by HSV1 can be divided into two phases: early and late host shut-off. The early host shut-off is seen in the presence of actinomycin (Fenwich and Walker, 1978) and can be induced by UV-irradiated HSV1 (Nishioka and Silverstein, 1977) indicating that a virion component is responsible for the phenomenon. This is known as virion host shut-off (vhs) and has been mapped to UL41 (Kwong *et al.*, 1988, 1989). EHV1 also contains a counterpart gene of HSV1 UL41 (gene 19). However, whether it also functions as a vhs gene is unknown. During the early host shut-off cellular polyribosomes are disaggregated (Sydiskis and Roizman, 1967) and host mRNAs subsequently degraded (Schek and Bachenheimer, 1985). The early host shut-off is followed by late shut-off which is conditional upon the synthesis of viral RNA and proteins. This shut-off is responsible for the enhanced reduction of host protein synthesis and for a decrease in cellular DNA synthesis (Fenwick, 1984). However, the mechanism of this effect is still unclear.

Available information has shown that EHV1 infection also causes inhibition of host DNA synthesis at an early stage of infection and eventually can shut off 95% of host DNA synthesis (Randall and Walker, 1963; O'Callaghan *et al.*, 1968a). At early times, EHV1 inhibits dramatically cellular RNA synthesis in L-M and human carcinoma cells (O'Callaghan, *et al.*, 1968b; Lawrence, 1971). It has been demonstrated that host cell replication is shut off a few hours after infection, but total protein synthesis initially continues at a linear rate which suggests a smooth transition from the synthesis of host protein to viral protein during the early stage of infection. However, the rate of total protein

synthesis began to decrease between 5 and 6 hours after infection and progressively decreases during the course of infection (Caughman *et al.*, 1985; Lawrence, *et al.*, 1971).

1.7.3. Replication of viral DNA

EHV1 DNA replication in EHV1 infected L-M cell is first detected 4-6 hours post-infection and is complete by 16-18 hours post-infection (O'Callaghan et al., 1968a, b). EHV1 DNA replication has also been studied in Syrian hamsters where it begins at 3-4 hours post infection and increases until the animal dies at 12-14 hours post-inoculation (O'Callaghan et al., 1972). The mechanism of herpesvirus DNA replication is not fully understood. In HSV, electron microscopic analysis shows that DNA molecules circularise after infection (Friedman *et al.*, 1977; Hirsch *et al.*, 1977). This has been found to result in the direct ligation of the terminal 'a' sequence (Jacob and Roizman, 1977; Davison and Wilkie, 1983b). At late times after the infection, large head to tail concatamers lacking termini appear which are hypothesised to be generated by a rolling circle mechanism (Jean and Ben-Porat, 1976; Jacob *et al.*, 1979). The initial sites of viral DNA replication have been demonstrated to be virus specific structures located within the infected nucleus and which are induced by infection (Quinlan *et al.*, 1984). These structures grow and coalesce as infection proceeds (Rixon *et al.*, 1983).

1.7.3.1. Origin of DNA replication

DNA sequence analysis of EHV1 has shown that the genome contains three candidates for origins of DNA replication (oriL and 2 oriS; Telford *et al.*, 1992). The first EHV1 origin of replication (oriS) had been previously identified by analysis of EHV1 defective interfering (DI) particles (Baumann *et al.*, 1989). Since DI DNA must contain cis-acting DNA sequences, such as DNA replication origins, the conserved regions in the DI DNA were examined by plasmid based transfections. A 200 bp fragment within 0.83-0.85 map units was shown to contain an origin of replication activity. Sequence analysis indicated that this region has significant homology to HSV1 and VZV origins of DNA replication. It contains, adjacent to an A+T rich palindrome, a 9-bp (CGTTCGCAC) sequence which is

identical to that recognised by the origin-binding protein encoded by the HSV1 gene UL9 (Elias and Lehman, 1988; Stow, 1992; Stow and Davison, 1986). As oriS is located in the short repeat region (IRs/TRs), the EHV1 genome contains two copies of oriS. A third EHV1 origin of replication (oriL) is located between genes 39 and 40, homologues of UL22 and UL21 in HSV1. Sequence analysis showed that it also contained the 9-bp sequence (CGTTCGCAC), adjacent to an A+T rich palindrome, which is conserved in all human herpesvirus origins of replication (Telford *et al.*, 1992; Robertson *et al.*, 1991). It has not yet been demonstrated to function as an EHV1 DNA replication origin.

1.7.3.2. Gene products required for replication

During the course of HSV1 infection, several virus specific gene products are involved in viral DNA replication. Some of them are associated with the virus particle and others have been identified in virus infected cells. A set of seven genes (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) products were found to be both necessary and sufficient for origin dependent DNA replication (McGeoch *et al.,* 1988a; Wu *et al.*, 1988; Stow, 1992). The counterparts of these genes are also present in EHV1 genome, genes 57, 54, 53, 31, 30, 18 and 7 (Telford *et al.*, 1992).

These gene products are the follows:

(1) A HSV1 encoded polymerase (Pol) has been demonstrated which is distinguishable from the host cell polymerase (Knopt, 1979; Haffey *et al.,* 1990). This enzyme is encoded by UL30 (Quinn and McGeoch, 1985). A polypeptide encoded by UL 42 has been found to act as an accessory subunit of DNA polymerase (Gottlieb *et al.*, 1990). UL42 can increase the ability of Pol to synthesize longer DNA products (Gottlieb *et al.,* 1990). The DNA polymerase has an intrinsic 3'-5' exonulease/RNase activity similar to *E.coli* PolI (Crute and Lehman, 1989a). Both UL30 and UL42 are essential for virus growth (Aron *et al.,* 1975; Marcy *et al.,* 1990; Johnson *et al.,* 1991).

EHV1 genes 30 and 18 are homologues of UL30 and UL42. Allen *et al,* (1977) demonstrated that EHV1 also induced a viral specific DNA polymerase which has different

immunological specificity from the host enzyme and a high salt requirement for maximal activity. It is far more sensitive to phosphonoacetic acid (PAA) than the enzyme encoded by HSV1 (Honess and Watson, 1977; Allen *et al.,* 1977).

(2) A HSV1 induced DNA-dependent ATPase has been identified(Crute *et al.,* 1988). Three products with M_rs of 130, 97 and 70 KD encoded by genes UL52, UL5 and UL8 respectively have been identified in the purified complex which contains the activity (Crute *et al.,* 1989b; McGeoch *et al.,* 1988b). The UL52 and UL5 genes have been found to enable close co-ordination of the priming of the lagging strand DNA with unwinding of the replication fork. The UL8 protein is important for efficient nuclear uptake of the helicase-primase complex (Calder *et al.,* 1992). However, the precise role which each subunit plays in these activities is unclear. The EHV1 homologues are genes 7, 57 and 54, resepectively.

(3) The major HSV1 DNA binding protein (mDBP) has been demonstrated to preferentially bind to single stranded DNA (Bayliss *et al.,* 1975). This protein is encoded by HSV gene UL29. It has characteristics of a helix destablizing protein. It binds to ssDNA and enhances the denaturation of a polydeoxyadenylic acid-polydeoxythymidylic acid duplex (Ruyechan, 1983; Ruyechan and Weir, 1984). It is also likely that this protein specifically interacts with other replication proteins and plays a role in organising DNA replication proteins (de Bruyn Kops and Knipe, 1988; Bush *et al.*, 1991). The EHV1 homologue is gene 31.

(4) A HSV1 origin binding protein is encoded by gene UL9 which has been found to specifically bind to an origin of replication (Olivo *et al.,* 1988). It has been demonstrated that UL9 also contains a helicase activity which is required for UL9 function (Stow, 1992). UL9 plays an essential role in DNA replication (Elias and Lehman, 1988; Weir and Stow, 1990). The EHV1 homologue is gene 53

1.7.4. Transcriptional features of viral genes

Most of the knowledge concerning regulation of herpesvirus gene transcription and expression has been obtained from studies of herpes simplex virus (HSV). It has been found that HSV1 gene expression is a co-ordinately regulated and sequentially ordered process in which at least three classes of viral gene products, termed alpha- ,beta- and gamma- (Honess and Roizman, 1974, 1975) or immediate-early(IE), early (E) and late (L) proteins (Clements *et al.*, 1977) were defined on the basis of their temporal appearance during the course of infection and the metabolic requirements for either *de novo* protein or DNA synthesis. Transcription of the immediate early (IE) gene occurs without *de novo* protein synthesis and is mediated by host RNA polymerase II (Mackem and Roizman, 1980, 1981; Costanzo *et al.*, 1977). Initiation of early (E) genes expression requires one or more functional IE polypeptides and occurs prior to and is independent from viral DNA synthesis. Late (L) gene expression is dependent on or is dramatically enhanced by the onset of viral DNA replication. Thus late proteins can be differentiated from IE and early proteins on the basis of their reduced production in the absence of viral DNA replication during infection. This "cascade" expression pattern of HSV1 genes is, however, more complex than a simple 3-fold one (Wagner, 1985). The late genes can be subclassified into either leaky-late, which requires 3 to 5 hours of protein synthesis before expression, or true-late which are also dependent on viral DNA replication (Weinheimer and McKnight, 1987; Jones and Roizman, 1979; Harris-Hamilton and Bacheheimer, 1985). In general, the IE genes code for transcriptional regulatory proteins, E genes are involved in DNA replication and L genes are structural and assembly proteins.

Similar, although apparently not identical, patterns of temporal regulation have been demonstrated for several other herpesviruses such as HCMV (Blanton and Tevethia, 1981; Wathen and Stinski, 1982) and PRV (Ihara *et al.*, 1983). EHV1 gene expression has been found to be temporally regulated in a manner (IE, E and L) similar to that demonstrated for HSV (Caughman *et al.*, 1985; Cohen *et al.*, 1975b; Gray *et al.*, 1987a, 1987b).

1.7.4.1. Transactivation of Immediate Early Gene Expression by the EHV1 Gene 12 product, homologue of HSV1 Vmw65

Several experiments have shown that a virion component located outside the nucleocapsid is responsible for the regulation of HSV1 immediate early gene expression. Using a series of co-transfection experiments, Campbell *et al.*, (1984) identified that this transactivating virion component was the major tegument protein, Vmw65 (also know as VP16), which is essential for the assembly of progeny virions (Ace *et al*., 1988).

A consensus AT-rich cis-acting sequence, TAATGARATTC (R=purine) was shown to be responsible for mediating the responsiveness to transactivation by Vmw65 (Mackem & Roizman, 1982a,b,c; Cordingley *et al.,* 1983). This consensus sequence was found to be present in the far upstream regions of all the HSV1 IE genes (Murchie & McGeoch 1982; Mackem & Roizaman, 1982a,b; Whitton & Clements, 1984). Vmw65 itself does not possess any intrinsic DNA binding activity, neither specifically for the TAATGARAT element nor non-specifically for double stranded DNA (Marsden *et al.*, 1987, Preston *et al.*, 1988). However, studies of the interaction between Vmw65 and cellular proteins revealed that Vmw65 formed a specific complex, called an immediate early complex (IEC), with the TAATGARAT motif, only in the presence of cellular proteins under conditions that support the binding of these cellular factors to DNA (McKnight *et al.,* 1987; O'Hare & Goding, 1988; Preston *et al.,* 1988). The protein Oct-1(NFIII,OTF-I), present in a wide range of cell types, has been demonstrated to be one of the cellular component of the complex (Ephrussi *et al.,* 1985; Gerster and Roeder, 1988). It has been found that Vmw 65 is composed of two distinct and functionally separable domains. The C-terminal 78 amino acids enriched for negatively charged amino acid residues specify the transactivation activity, whereas the N-terminal region is essential for the IEC formation.

EHV1 gene 12 has been shown to encode a functional homologue of HSV1 Vmw65, despite its predicted lack of a carboxy terminal acidic domain (Lewis *et al.*, 1993; Purewal *et al.,* 1994). Elliott and O'Hare (1995) found that EHV1 gene 12 could activate the EHV1

IE promoter and the HSV1 IE110 promoter efficiently. Closer examination of the sequence between -360 and the RNA start site (+1) of the EHV1 IE promoter (Harty *et al.,* 1989) revealed the presence of four potential octamer binding motifs, E1, E2, E3 and E4 (Elliott and O'Hare 1995). E1 and E2 diverge by only one residue from the canonical octamer consensus site (ATGCAAAT). E3 containing a POU-specific site diverges by a single base in the 5' region (Sturm and Herr, 1988; Verrijzer *et al.,* 1990). The *in vitro* complex formation assay has shown that the four octamer binding motifs could form a strong binary complex with Oct-1 and the gene 12 product (Elliott and O'Hare, 1995). Like Vmw65, the EHV1 gene 12 transactivator utilises octamer DNA binding sites E1 and E4 to interact with the EHV1 IE gene promoter and stimulate expression of the EHV1 IE gene (Elliott and O'Hare, 1995).

1.7.4.2. Immediate Early Gene Expression

Gray *et al.*, (1987 a) demonstrated that during EHV1 productive infection sequences within the short component of the viral genome are the first to become transcriptionally active. A 6.0 kb IE transcript which mapped to the inverted repeat sequences (m.u. 0.78-0.83 and 0.95-1.0) was identified in the absence of protein synthesis. DNA sequence analysis of the regions identified an ORF (gene 64) consisting of 1487 codons (4461bp) with high homology to HSV1 Vmw175 (Grundy *et al.*, 1989; Telford, *et al.*, 1992). This predicted IE ORF contains a high G+C content (74.3%) in its codon usage. A similar high G+C content (81.5%) has also been found in the HSV1 IE gene, Vmw175 (McGeoch *et al.*, 1986 b). Sequence analysis has mapped two sequences resembling signals for transcription initiation (TATA boxes; McKnight and Kingsbury, 1982) upstream of the initial ATG of the EHV IE gene, at -654 and -319. Transcription originating from this region would result in a leader sequence of approximately 600 bases for the IE gene. S1 nuclease and primer extension analysis demonstrated that a 372-nucleotide sequence is removed by splicing, to produce a 220 bp leader sequence upstream of the gene 64 start codon (Harty *et al.*, 1989). The HSV-1 ICP4 transcript also has a 250 base leader (McGeoch *et al.*, 1986 b; Rixon *et al.,* 1982). These sequence features of the putative EHV1 IE gene demonstrated high amino acid

homology and comparable location of the gene to the two previously characterised IE proteins, HSV1 ICP4 and the VZV gene 62 protein (McGeoch *et al.,* 1986b; Paterson and Everett, 1988; Davison and Scott, 1985).

Four abundant IE polypeptides, designated IE1, IE2, IE3 and IE4, have been detected in infected cells released from a protein synthesis block (Caughman *et al.,* 1988), but they have never been detected in infected cells under normal conditions. The same situation has also been observed for expression of PRV 180 KD IE protein (Ihara *et al.*, 1983), which was not detected in extracts from unblocked wild-type PRV infections. The presence of a single 6 kb IE mRNA and the finding of four IE polypeptides raised the question whether the four IE proteins were related proteins from the same gene. Characterisation of the four IE proteins revealed that the EHV1 IE proteins (IE1-4) are related structurally and antigenically and appear to be either produced simultaneously or processed to yield the individual forms immediately (Caughman *et al.*, 1988). *In vitro* translation experiments have demonstrated that a family of IE proteins is generated *in vitro* from the 6.0 kb mRNA size class and that these translated IE proteins correspond in overall size and antigenicity to those synthesised in infected cells (Caughman *et al.*, 1985). These findings demonstrated that a family of EHV IE proteins including IE1-4 are translated from the 6.0 kb IE mRNA size class (Caughman *et al.,* 1985). However, the mechanism involved in their generation is not clear.

Using an EHV1 IE gene expression vector and recombinant target promoter-CAT reporter constructs, Smith *et al.* (1992) demonstrated that the EHV1 IE gene was capable of both transactivation and trans-repression. The EHV1 IE gene , expressed from the predicted IE ORF, was found to be able to independently activate the EHV1 TK promoter by as much as 60-fold and could activate the HSV-tk promoter by approximately 17-fold. It was also able to transactivate two EHV1 beta- and gamma-promoters; however, in this case there is a requirement for additional gene products from the EHV1 XbaI G (map unit 0.01-0.06) restriction fragment which contains EHV1 gene 5, the homologue of UL54 (ICP27) of

HSV1. In addition, it has been demonstrated that the EHV1 IE protein can repress expression from its own promoter (Smith *et al.*, 1992). Two cis-acting autoregulatory elements have been mapped to a region within -288 to +73 of the IE transcription initiation site with a Vmw175-like binding site 5'-ATCGTC(N4)CGCGG-3' sequence spanning the transcriptional start of the EHV1 IE gene (Smith *et al.*, 1992). Recently, Kim *et al.*, (1995) demonstrated that the IE protein could bind to sequences from -11 to -14 of the EHV1 IE transcription initiation site. They also found that the IE protein binds to a degenerate version of the consensus sequence 5'-ATCGT-3' in EHV1 early and late promoters in which only C or G residues are essential for the binding (Kim *et al.*, 1995). These findings suggest that formation of a specific complex between the EHV1 IE protein and EHV1 gene promoters including its own promoter could activate early and late gene transcription and autoregulate its own expression .

1.7.4.3. Early and late gene expression

Caughman *et al.* (1985) have characterised the EHV1 infected cell polypeptides (ICPs) during the course of infection in the presence or absence of an inhibitor of virus DNA synthesis. At least 34 EHV1 ICPs ranging in apparent molecular weights from 16.5 KD to 213 KD were detected. Twelve were classified as late proteins since inhibition of viral DNA replication resulted in significantly reduced synthesis and/or accumulation of these polypeptides; 17 were classified as early proteins since their synthesis is not reduced by the inhibition of viral DNA replication and at least three polypeptides (137KD, 74KD and 31.5KD) accumulate in large amounts in phosphonoacetic acid (PAA) treated cultures compared to cultures in which the infection process was unimpaired.

EHV1 genes (63, 5 and 65) that are homologous to three HSV1 IE genes (ICP0, ICP27 and ICP22) are transcribed as early genes (Holden *et al.*, 1992; Telford, 1992; Smith *et al,* 1993). Sequence analysis reveals that EHV1 gene 63 possesses a low level of homology to HSV ICP0, but it retains the zinc finger motif (Zhao *et al.*, 1992). EHV1 gene 5 is the homologue of ICP27 of HSV1 and its product has been found to function synergistically

with the IE gene product to transactivate late promoters (Smith *et al.*, 1992, 1993; Zhao *et al.*, 1995). It can upregulate the expression of the EHV1 IE promoter by itself although the gene 5 product by itself does not efficiently transactivate early or late promoters (Zhao *et al.*, 1995). EHV1 gene 65, homologous to the HSV1 US1 gene (ICP22) has been found to be transcribed as a major early transcript of 1.4 kb and a minor late transcript of 1.7 kb (Holden *et al.*, 1992). Gene 65 encodes a series of proteins with a M_r of 42 to 47 KD which are localised to the nucleus and present in the EHV1 virion (Holden *et al.*, 1994). Using a gene 65 expression vector cotransfected with a EHV1 chimerical promoter-CAT reporter construct and EHV1 effector plasmids, Holden *et al.*, (1994) demonstrated that the gene 65 protein also minimally trans-activates EHV1 promoters, but acts synergistically with the gene 5 product to transactivate EHV1 promoters; it also enhances transactivation of early promoters by the IE protein and enhances the transactivation of both early and late promoters by the IE and gene 5 proteins.

1.7.5. Assembly and egress of virions

It has been shown that pharmacological agents that impinge on the secretary organelles, such as monensin (Johnson and Spear, 1982) and tunicamycin (Pizer *et al.*, 1980) interfere with formation and release of HSV from infected cells. Furthermore, the mutant cell line gro29, which is defective in protein secretion, is unable to release virions when infected with HSV 1, despite normal viral gene expression. These studies suggest that the secretory pathway is critical to HSV release. Other cell lines defective in a variety of enzymes required for processing of N-linked oligosaccharides have been shown to produce less infectious virus than normal cells when infected with HSV1, suggesting specific oligosaccharide chains contribute to efficient viral maturation and egress (Campadelli-Fiume *et al.*, 1982; Serafini-Cessi *et al.*, 1983). The cellular localization of assembly, maturation and release of herpes viruses has not been mapped in detail. Nevertheless, evidence has been obtained in several systems that the nucleocapsid is first assembled as a DNA-free precursor capsid, into which viral DNA is subsequently packaged (Roizman and Sears, 1990; Roizman and Furlong, 1974; Schwartz and Roizman, 1969). In general,

herpes viruses are unique among enveloped viruses in that the primary site of envelopment appears to be at the nuclear membrane especially the inner lamella, though the process may occur at nuclear matrix, cytoplasmic, or plasma membrane sites (Ben-Porat and Kaplan, 1972; Dargen, 1986; Darlington and Moss III, 1968; Johnson and Spear, 1982; McCracken and Clarke, 1971; Morgan *et al.,* 1954; O'Callaghan and Randall 1976; Poliquin *et al.*, 1985; Spear, 1984; Stackpole, 1969). Johnson and Spear (1982) suggested a model for HSV1 envelopment and viral egress by which HSV1 virions were first enveloped at the inner nuclear membrane, where they acquired viral glycoproteins lacking Golgi modification. Subsequently, these immature virions were transported to the Golgi apparatus, where precursor glycoproteins were modified as the enveloped particles moved through the Golgi stacks.

Darlington and Moss (1968) demonstrated that the envelopment processe for EHV1 strain Ky D are the same as that of HSV1(strain H4) and PRV. Envelopment of virus at the inner nuclear membrane could be seen in sections coincident with or shortly before the increase in release (5 to 8 hours after infection). When viral capsids, with or without DNA, came into contact with the inner membrane, this membrane became noticeably thicker at the point of contact. The particles became completely enclosed in this thickened membrane and appeared to pinch off leaving the nuclear membrane intact in a manner analogous to the formation of pinocytic vesicles at the plasma membrane. After release from the nucleus, the enveloped particles were found in the cytoplasm in vacuoles, sometimes enclosed in an additional membrane containing several particles which was apparently derived from cytoplasmic elements (Darlington and Moss 1968).

Whealy *et al.*, (1991) have investigated PRV viral glycoprotein processing and virus egress using brefeldin A (BFA), which had little effect on initial synthesis and cotranslational modification of viral glycoproteins in the endoplasmic reticulum (ER), but disrupted subsequent glycoprotein maturation and export into post-Golgi cellular compartments (Doms *et al.*, 1989; Lippincot-Schwartz *et al.*, 1989; Misumi *et al.*, 1986). Their results

showed that envelopment of PRV involved initial acquisition of a membrane by budding of capsids through the inner leaf of the nuclear envelope followed by deenvelopement and release of these capsids from the ER into the cytoplasm in proximity to the trans-Golgi. The released capsids then acquire a bilaminar double envelope containing mature viral glycoproteins at the trans-Golgi. The resulting double-membrane virus is transported to the plasma membrane, where membrane fusion releases a mature, enveloped virus particle from the cells.

A view of VZV maturation has recently been produced by Harson and Grose (1995) who investigated the entire route of VZV egress from the nucleus to the outer cell membrane. Based on observations from transmission electron microscopy(TME) at intervals post infection, they provided a hypothesis of VZV egress. The nucleocapsid passed through the inner nuclear membrane and acquired its initial envelope. The enveloped particle then travels through the perinuclear space where it is engulfed within a vacuole formed in a similar manner to the premelanosome. A vacuole containing one or more viral particles is pinched off and resides within the cytoplasm. After the viral glycoproteins are processed during transit in the Golgi, they collect within transport vehicles, in a manner similar to tyrosinase, and travel to fuse with a smooth endoplasmic reticulum (SER)-derived vacuole containing nascent virions. Once viral glycoproteins, including VZV gH-gL complex, have entered the vacuole, the vacuole can fuse with one or more neighbouring vacuoles to form a larger vacuole. In turn, the vacuole exhibits the exocytic properties of a melanosome, whereby it can travel through the cytoplasm and fuse with the outer-cell membrane (Harson and Grose, 1995).

Mutants in a number of HSV1 genes have been shown to affect the egress of virus from infected cells (Baines *et al.*, 1991; Campadelli-Fiume *et al.*, 1990; Desai *et al.*, 1988; Baines & Roizman, 1992). These genes encode gD, gH, gL and the UL11 and UL20 proteins. Desai *et al.* (1988) found that a temperature-sensitive mutant which contains an amino acid substitution in gH entered cells efficiently at the non-permissive temperature

and replicated to give nearly normal yields of intracellular infectivity. The intracellular virions contain, predominantly, an immature form of gH, while excreted virions were devoid of gH and were not infectious. Additionally, no gH was found on the surface of infected cells. On the other hand, virions excreted at the permissive temperature contain mature gH and were infectious. No loss of gH results from incubation of these virions at the non-permissive temperature. This data indicats that gH is an essential component of infectious virions.

MacLean *et al.* (1989) identified that the UL11 gene of HSV1 encodes a myristylated protein. They also found that a UL11 deletion mutant in which 40% codons of coding sequence(109bp of the 288bp coding sequence) was disrupted by a lacZ insertion grows about 20-fold less well than wild-type and its revertant, and produces smaller plaques. Baines and Roizman, (1992) demonstrated that a deletion mutant lacking 61% of the codons (176bp of the 288bp coding domain) of HSV1 UL11 gene reached titres 30-250 fold lower than those obtained from cells infected with wild-type virus. Electron microscopic analysis indicated that both full and empty capsids accumulated in the nuclei, juxtaposed with the inner lamellae of the nuclear membranes. There was a >1,00-fold decrease in the amount of infectious extracellular virus released from Vero cells infected with the deletion mutant compared with that from cells infected with wild-type virus. Furthermore, the onset of release of infectious virus from cells infected with the UL11$^-$ virus was significantly delayed. These data indicated that the absence of the UL11 gene causes a reduced capacity to envelope and transport virions into the extracellular space.

Baines *et al.* (1991) have isolated a HSV1 deletion mutant in which 53% of the UL20 gene ORF had been deleted. It was found that the yields of UL20$^-$ virus were approximately 10- to 100-fold lower than those of wild-type virus in 143 tk$^-$ cell cultures. No infectious virus was produced in Vero cells by this mutant. Electron microscopic examination of Vero cells infected with the UL20$^-$ mutant revealed that enveloped and unenveloped capsids accumulated in the cytoplasm, possibly in the space between the inner and outer lamellae of

the nuclear membrane, and that virtually no virus was present in the extracellular space. This data indicates that UL20 encodes a protein with a hitherto unrecognised function which enables the transit of virions to the extracellular space. Avitabile *et al.* (1994) found that in Vero cells infected with UL20$^-$ virus, immature viral glycoproteins associated with virions sequestered in the space between the inner and outer nuclear membranes and only a limited amount of viral glycoproteins containing complex-type oligosaccharides with sialic acid are transported to the plasma membranes. In contrast to the observation in Vero cells, in infected 143TK$^-$ cells UL20$^-$ virions are exported to the extracellular space, although the yields of infectious progeny are lower than that of wild-type virus, and the amount of viral glycoproteins exported to the plasma membranes of cells infected with the wild-type parent or with UL20$^-$ virus are similar. These observation suggest that in Vero cells UL20 is required for the packaging of virions by the outer nuclear membranes into transport vesicles which carry the virions through the Golgi apparatus, where the glycoproteins are fully processed, to the extracellular space and for the transport of membrane-associated viral glycoproteins from the trans-Golgi compartment to the plasma membrane. This requirement is obviously cell-type-dependent.

Arsenakis *et al.* (1988) have found that a baby hamster kidney (BHK) cell line expressing gD (e.g. the BJ-1 clonal cell line) of HSV-1 strain F allows the wild-type virus to attach, but virus was internalized by endocytosis and degraded. Using this cell line a mutant (HSV-1(F) U-10) which was capable of infecting the BJ-1 clonal cell line was selected. Marker rescue and sequencing studies mapped the mutation to a fragment which encoded a small numbers of genes including the gD gene and demonstrated that in the mutant virus amino acid Leu-25 of gD was replaced by proline since exposure of the BJ-1 cells to the nonneutralizing monoclonal antibody AP7 which recognised an epitope of gD including Leu-25 rendered the cells infectible with wild-type virus (Minson *et al.*, 1986). Campadelli-Fiume *et al.* (1991) found that at late time point infection although the amount of virus recovered from HSV-1 (F)-10 -infected cells was 5- to 10- fold lower than those recovered from HSV1 (F)-infected cells, the amount of virus recovered from the

extracellular fluid was approximately 50-fold less than that recovered from extracellular medium of cultures infected with the wild-type parent. In addition, a large number of unenveloped capsids accumulated in the cytoplasm juxtaposed to the cytoplasmic membranes, suggesting that the process of envelopment of HSV-1(F)-U-10, is very slow and that virus egress from cells is impaired. These data indicate that several HSV1 gene products are involved in the different stages of virion formation and release from infected cells.

1.8. ANIMAL MODELS FOR THE STUDY OF EHV1 INFECTION

EHV1 infection is an infection of major importance, especially in the thoroughbred industry, and there is much interest in the development of effective vaccines or chemotherapeutic agents to prevent infection or disease, or to reduce virus shedding in infected animals. It therefore became essential to develop suitable animal models for studying the immune response to EHV1 infection, its pathogenicity and to evaluate the efficiency and safety of vaccines. For this reason, three laboratory animal models have been established.

1.8.1. A hamster model

The Syrian hamster has been used for studying EHV1 infection and aspects of immunity to EHV1 infection for a long time (Anderson and Goodpasture, 1942; Wilks and Coggins, 1977). Intraperitoneal (i.p.) challenge with a virulent isolate of EHV1 (strain Ky B) at a dose of $5x10^6$ pfu/hamster resulted in 100% mortality of infected hamsters by day 3 with peak levels of virus in the liver at between 48 and 60 h p.i. and in the lungs at 3 days p.i., indicating that the acute disease produced by EHV1 infection with hamsters dying in 3 to 5 days is most unlike the equine disease and the serious limitation of this model is that it does not permit full assessments of immune effector mechanisms. However, using this model Wilks and Coggins. (1977) demonstrated that passive transfer of immune splenocytes, peritoneal exudate cells or antiserum can protect the animal from EHV1 challenge.

Stokes *et al.* (1989) developed a subacute hamster model using EHV1 strain Ky D and DSN inbred hamsters. Animals infected by i.p. or intra-nasal (i.n.) administration of low titre (5x 10^5 pfu/animal) EHV 1 (Ky D) developed a self-limiting infection in which virus clearance was complete by day 6. I.p. inoculation of virus resulted in a systemic infection, virus being isolated from the major organs by day 4. while i.n. inoculation is more similar to the natural route of infection and has been used to administer other respiratory pathogens (King *et al.,* 1987). Following infection of DSN inbred hamsters either i.p. or i.n. with EHV1 strain KyD, virus was isolated from the livers of all animals in both groups by day 2 and consistently isolated from the liver, lung, heart, kidney and spleen of i.p. infected hamsters by day 4. The majority of virus was consistently isolated from the liver during infection. Infection resulted in the occasional death but usually animals were clinically affected for 4 days and then recovered. Virus clearance was apparent by day 6 p.i.

1.8.2. A murine model.

The pathogenesis of infection in hamsters differs quite strikingly from that in the natural host, eg the liver is a primary site of virus replication in the hamster and the histopathology does not resemble that observed in the natural respiratory disease. To avoid these disadvantages of the hamster model, a murine model was developed for studying the immunogenesis and pathogenesis of EHV1 (Awan *et al.*, 1990). New-born mice had previously been shown to be susceptible to EHV1 following intracerebral inoculation (i.c.) (Patel and Edington, 1983). Awan *et al.* (1990) demonstrated that female BALB/c mice (3-4 week old) are generally considered refractory to EHV1 infection and can provide a relevant model system for several features of the infection which are apparent in the horse. Mice are not only very convenient for study, but a wealth of information exists already on their responses to herpesvirus infections.

Two days following i.n. inoculation of EHV1 at a dose of 5x10^6 pfu/mouse, mice began to show abnormal signs and by day 3 all mice were hunched with ruffled fur and a

conspicuous weight reduction. Some mice showed signs suggesting a mild neurological involvement. Death occured from the fourth to the seventh day after inoculation, with approximately 50% of animals recovering from the infection and becoming clinically normal by day 10. Virus was consistently isolated from the nasal tissues, trachea and lungs from all mice tested during the first week after inoculation and occasionally from the CNS, eyes and liver. Virus titres reached a peak at 3 to 5 days after inoculation, coincident with the time of maximum clinical signs. Virus was cleared from the lungs and turbinate bones by day 12 after inoculation. Histological evidence of virus replication was observed in the lung after 24 h inoculation. Electron microscopy showed that alveolar and bronchiolar tissues cells contained signs of herpesvirus infection including disrupted chromatin; altered nuclear membrane; the presence of dense cored virions in the nucleus; and both cytoplasmic and extracellular enveloped particles. An important feature of EHV1 infection in its natural host is the viraemic spread of virus during the acute disease. A vireamia was also observed in EHV1 infected mice during the acute phase of the infection. Approximately 1 in $5x10^4$ blood cells were demonstrated to be infected.

Immune responses against EHV1 infection were also analysed for the presence of antibodies specific to EHV1 by means of an ELISA and virus neutralisation test. Following the i.n. inoculation of mice with EHV1 an active humoral and T cell immune response was detected. Using an ELISA antibody first appeared on the 12th day after inoculation with EHV1, peaked at day 25 and showed a marked fall by day 30. In contrast to HSV1, the antibody levels of EHV1 infected mice waned markedly on the last occasion tested (day 30) (Nash *et al,* 1985; Awan *et al.,* 1990). No neutralizing activity from mouse serum could be detected at any time after i.n. or i.p. inoculation with either live or inactivated virus, in contrast to the hyperimmune sera previously raised in mice by repeated subcutaneous (s.c.) injection of virus antigen together with adjuvant. A powerful skin reaction of the classical delayed type hypersensitivity (DTH) to injection of heat-inactivated antigen into the primary infected mouse skin suggested that cell-mediated responses are activated during the primary infection. These data indicated that the antibody response to EHV1 in the murine

model following i.n. inoculation was relatively poor with no neutralizing antibody against EHV1 present in serum, implying that local responses or cellular immunity are likely to be more important factors in protection from EHV1 infection. Azmi and Field (1993) have examined the cell-mediated response to EHV1 infection in the murine model by means of adoptive transfer of immune cells. They found that adoptive transfer of immune cells from mice primed by live or heat-inactivated EHV1 conferred an enhanced DTH response in recipients. Only the immune cells from mice previously primed with live EHV1 gave protection against infection, indicating an important role for specific cell-mediated responses in protective immunity to EHV1 infection.

1.8.3. Specific pathogen-free (EHV-free) foals

Colostrum-deprived specific pathogen-free (EHV-free) (SPF) foals are taken from their dams at birth and reared by hand using bovine colostrum (Chong *et al.*, 1991). They are free from EHV infection and also from maternal antibodies. They have proved to be invaluable in the study of EHV infection. Tewari *et al.* (1993) have examined the response of SPF foals to EHV1 infection. Following primary and secondary i.n. inoculations with EHV1 at a dose of 10^7 pfu/foal, five SPF foals showed clinical signs of primary infection including nasal excretion of virus, which continued for 11 days, and viraemia. On secondary infection, clinical signs were minimal and virus was recovered on only day 1 p.i., and vireamia was not detected (Tewari *et al.*, 1993). The serological response of SPF foals to EHV1 was observed (Tewari*et al.*, 1993). Both complement fixing and virus neutralizing antibodies against EHV1 were detected by day 15 p.i. and reached peak titres between 40-60 days p.i. The titre of complement fixing antibodies declined prior to secondary EHV1 infection, 12 weeks later. In contrast, virus neutralizing antibodies were sustained and remained at maximum levels until re-infection, 12 weeks later (Tewari *et al.*, 1993). Immune blotting using EHV1 antigens showed that following primary infection with EHV1, no specific antibodies against viral proteins were detected in primary infected SPF sera up to day 15, but by day 60-85 p.i. the sera could recognise four or five major EHV1 proteins with molecular weights 110/115 k, 78k, 58k, and 48k (corresponding to

gp10, gp14, gp18a and/or gp18b, and gp21/22a). On secondary infection with EHV1, 12 weeks after the primary inoculation no increase in intensity of the bands was seen and no new bands were detected by the secondary infected SPF sera (Tewai *et al.*, 1993). These observations showed that a primary i.n. inoculation with EHV1 resulted in a strong serological response recognising EHV1 specific antigens in SPF foals. Whilst reinoculation with the same virus produced a boost to EHV1 antibodies, no additional antigens were recognised.

1.9. VACCINES AND VACCINATION

Vaccination for the prevention of herpesviral diseases of the horse caused by EHV1 is based on the concept that naturally acquired immunity to respiratory diseases caused by EHV1 is a product of the immunogenic conditioning of repeated infections (Doll *et al.*, 1963); it seems clear that immunological responses critical to the development of protective immunity to infection or disease are induced specifically by the antigenicity of the envelope glycoproteins of the virus (Papp-Vid and Derbyshire, 1979); that immunity to reinfection is short lived (Bryans 1969; Von Steinhangen, 1988); and that immunity to reinfection against abortigenic infection is required from the onset of the sixth month of gestation until term (Doll *et al.*, 1963). Two vaccines have been in wide-spread use in the United States; one is a live virus product attenuated by multiple passages in swine cell cultures followed by passage in equine cells (Burki *et al.*, 1990). It was originally recommended as a preventive vaccine for abortigenic infection by EHV1. Now it is limited to use for respiratory disease (Burki *et al.*, 1990). The second vaccine used in the U.S.A. is a formalin inactivated, adjuvanted formulation of an attenuated strain of EHV 1 (Bryans, 1978; Bryans, and Allen, 1982; Burrows *et al.*, 1984). Since the inactivated vaccine was made available to veterinarians, the mean incidence of EHV1 abortigenic infection in central Kentucky during a six year period declined from 7.4/1000 pregnancies to 2.3/1000 (range 1.0- 3.1/1000) (Burki *et al.*, 1990; Burrows *et al.*, 1984). Although the overall incidence of the disease decreased, both single and multiple abortions continued to occur in herds vaccinated with either the inactivated or the modified live virus vaccines during this period indicating

incomplete protection. The attenuated live vaccine can lead to the establishment of latent infection which can be reactivated by wild-type (WT) EHV1 infection. It can also cause recrudescence in wt EHV1 infected horses (Allen and Bryans, 1986; Onions, 1991).

Therefore, several attempts have been undertaken to improve the efficiency of vaccines. These attempts include developing more suitable animal models for studying the host immune responses to EHV1 infection and pathogenesis and constructing more efficient and safe vaccines such as attenuated deletion mutants and subunit vaccines comprising expressed virus glycoproteins. Some have been tested for evidence of their suitability as potential vaccine candidates on the established animal models. A thymidine kinase-deficient (TK^-) mutant of EHV1 was tested in the mouse model and SPF foals for infectivity, pathogenicity and induction of immunity to EHV1 infection (Slater *et al.*, 1993). Compared to wild-type virus, the TK^- mutant, PR1, produced markedly mild clinical disease following both i.n. and i. c. inoculation, and, in particular, no mortality occurred. PR1 did however establish productive infections following i.n. inoculation. High titres of virus were recovered from target organs although virus did not persist for as long as wt EHV1 and no viraemia was detected. In contrast to that with wt EHV1, the serological response was very weak in PR1 infected animals. However, primary i.n. infection with wt EHV1 protected against subsequent challenge with wt EHV1 5 weeks later (Slater *et al.*, 1993). PR1 could also not be reactivated by administration of dexamenthasone and could not be co-cultivated from any tissues of the ponies infected with PR1 (Slater *et al.*, 1994). A deletion mutant (ED71), which is a EHV1 gene 71 negative mutant and is demonstrated to be defective in entry into cells, egress from cells and subsequent transmission from cell to cell (Sun and Brown, 1994; Sun *et al.*, 1996), was also tested in the murine model for pathogenesis and immunogenesis (Tim Fitzmaurice, personal communication). The result showed that in the primary infection ED71 was attenuated and conferred good protective immunity to subsequent EHV1 challenge, both in reduction of virus recovered from tissues and numbers of animals found positive for virus.

Some EHV1 glycoproteins (subunit vaccines) have also been expressed and tested in animal models for stimulating the protective immunity against EHV1 infection. Tewari *et al.* (1994) tested host immune responses following immunization with extracts of insect cells infected by baculoviruses expressing EHV1 gD and gH. In contrast to the gH recombinant infected cells, the gD recombinant infected cells produced neutralizing antibodies to EHV1 and a protective immunity against subsequent EHV1 challenge, determined by accelerated clearance of virus from the target organs in the respiratory tract. In the mouse model both glycoproteins induced delayed-type hypersensitivity and lymphoproliferation to EHV1 antigen. Osterrieder *et al.* (1995) also tested various formulations of recombinant glycoprotein gp14 (gB) in a murine model. They found that only VLP-gp14, a truncated gp14 expressed as a fusion protein with human immunodeficiency virus type 1 gag to produce virus-like particles (VLP) in insect cells induced both a good humoral and a prominent delayed-type hypersensitivity (DTH) immune response to EHV1.

CHAPTER 2: MATERIALS AND METHODS

2.1. MATERIALS.

2.1.1. Cells

The following cell lines were used in this study.

Baby hamster kidney 21 clone 13 cells (BHK21/C13)
Equine dermal cells (NBL-6)
Rabbit kidney cells (RK13)
Human foetal lung fibroblasts (HFL)
African green monkey fibroblasts (CV-1)
Mouse embryo fibroblasts (3T6)

2.1.2. Viruses

Equine herpesvirus type 1 (EHV1)strain Ab4p (Telford *et al.*, 1992) was used as the wild-type virus. Virus stocks were prepared in NBL-6 cells and titrated on BHK21/C13 cells. Herpes simplex virus type 1 strain 17^+ (HSV1 17^+) (Brown *et al.*, 1973) was used as a comparative virus in some experiments.

2.1.3. Cell culture media

BHK21/C13 cells and HFL cells were grown in Eagle's medium (Gibco) supplemented with 10% new-born calf serum (Gibco), 10% (v/v) tryptose phosphate broth, 200mM L-glutamine (Gibco), 10,000 IU/ml penicillin and 10,000UG/ml streptomycin (Gibco). This is referred to subsequently as ETC10.

Variations on the basic growth media were:

PIC	Phosphate-free Eagle's medium containing 1% newborn calf serum
EMC10%	Eagle's medium containing 1% carboxymethyl cellulose and 10% newborn calf serum.
Emet/5C2	Eagle's medium containing one-fifth the normal concentration of methionine and 2% calf serum.
Eglu/10C2	Eagle's medium containing one-tenth the normal concentration of glucose and 2% calf-serum

Dulbecco's Modified Eagle's medium (Gibco) supplemented with 10% foetal calf serum (Gibco), 200mM glutamine (Gibco), 10,000 IU/ml penicillin and 10,000 UG/ml streptomycin (Gibco) was used for growth of 3T6, NBL-6, RK13 and CV1 cells.

2.1.4. Experimental animals

Ten female New Zealand white rabbits (weight : 1000g-1500g; Bantin and Kingman or Harlan U.K. Ltd.) were used in this study.

2.1.5. Bacterial strains

Escherichia coli strain NM522 [re CAT, sup E,thi, (lac-proAB), hsd 5(r^-, m^-), (F' pro AB, lac192 M15)] (Gough and Murray,1983), JM109 [supE,thi, (lac-pnAB), (F'traD36, proAB, lac192 M15] (Yanisch-Perron *et al.,* 1985) and strain XL1-Blue^r {[supE444hsdR17 recA1 gyrA46 thi relA1 alc] F'proAB+ lacZΔM15 Tn 10(tet^r)} were used for transformation of recombinant plasmids and expression of fusion proteins.

2.1.6. Bacterial growth media

The media used for bacterial growth were:

L-broth	10g/l NaCl, 10g/l bactotryptone (Difco), 5g/l yeast extract.
L-broth agar	L-broth containing 0.5% (w/v) bactoagar (Difco).
2xYT	85mM NaCl, 16g/l bactotryptone (Difco), 5g/l yeast extract.

2.1.7. Oligonucleotides

Synthetic oligonucleotides were synthesised in the Department of Virology by Dr J. McLauchlan, using a model 8600 Biosearch multiple column DNA synthesiser.

2.1.8. Plasmids

pUC19 (Yanisch-Perron *et al.*, 1985) was used for cloning and subcloning fragments from the EHV1 genome.

pUR278 (Rüther & Müller-Hill, 1983), a ß-galactosidase gene fusion expression vector, was used for construction of EHV1 fusion protein expression plasmids.

pCMV10 used for construction of the EHV1 gene 67 expression plasmid is a eukaryotic expression vector (Stow *et. al.,* 1993).

2. 1.9. Antibodies

P19, a monoclonal antibody against EHV1 glycoprotein gp300 (Whittaker *et al.*, 1992).

Monoclonal anti-a-tubulin, Sigma.

Monoclonal anti-actin (amoeba), Sigma.

Anti-rabbit IgG (whole molecule) Fluorescien Isothicanate (FITC) conjugated-antibody, Sigma.

Anti-mouse IgG (whole molecule) tetramethylrhodamine (TRITC) conjugated-antibody, Sigma.

Horse neutralizing antiserum against EHV1, kindly supplied by Dr. Neil Edington.

2. 1. 10. Enzymes

Restriction enzymes, Vent polymerase, T4 DNA ligase, T4 polynucleotide kinase and calf

intestinal alkaline phosphatase (CIP) were obtained from either Bethesda Research Laboratories (BRL), New England Biolabs or Boehringer Mannheim Corporation. Lysozyme, Deoxynuclease I and Ribonuclease A were obtained from Sigma Chemical Co.

Glycosidases:

a. N-glycanase [peptide-N-4 (N-acetyl-ß-glucosaminyl) asparagine amidase], Genzyme.

b. Neuraminidase (acetylneuraminyl hydrolase), Sigma.

c. ß-N-acetylhexosaminidase (2-acetamido-2-deoxy-ß-D-glucoside-acetamidodeoxyglucohydrolase), Sigma.

d. L-fucoside fusohydrolase, Sigma.

e. ß-galactosidase (ß-D-galactoside galactohydrolase), Boehringer Mannheim.

2. 1. 11. metabolic Inhibitors

Tunicamycin, Sigma

Monensin, Sigma

Brefeldin A, Sigma

Nocodazole (Methyl-(5[2-thienycarbonyl]-1 H-benzimidazol-2yl)-Carbamate), Sigma

2. 1. 12. Radioisotopes

All radioisotopes were supplied by Amersham International plc. They had the following specific activities:

	specific activity
^{32}P-Orthophosphate	3000Ci/mmol
^{35}S Methionine	>1000 Ci/mmol
^{14}C glucosamine	50 mCi/mmol
^{3}H mannose	10 to 20 Ci/mmol
UDP-^{14}C galactosidase	270mCi/mmol
5' [^{32}P] dNTPs	3000Ci/mmol
[^{35}S] dATP	4000Ci/mmol

2.1.13. Chemicals

The chemicals used were of analytical grade and most of these were supplied by either BDH chemicals, UK or Sigma Chemical Co. Exceptions were:

Acetic acid glacial	May and Baker Ltd., Dagenham, England.
Ampicillin sodium B.P. (Penbritin)	Beecham Research
APS and TEMED	Bio-Rad Laboratories.
Boric acid and glycerol	PROLAB.
Caesium chloride and acrylamide	Koch Light Ltd. (Suffolk, England)
2'-Deoxynucleotide-5'-triphosphate (dNTPs)	Pharmacia
Isopropylthio-b-D-thiogalactoside (IPTG)	Bethesda Research Laboratories (BRL).

Dimethylsulphate	Flukw AG, Buchs SG, Switzerland
Dimethylsulphoxide (DMSO)	Koch-Light Laboratories, England
Ficoll	Pharmacia, Uppsalla, Sweden
Glutaraldehyde	Agar Aids, Cambridge, England
Proteinase K	Boehringer Mannheim GmbH., W. Germany

2.1.14. Commonly used buffers and solutions.

Alkaline lysis solution I	10mM EDTA, 50mM glucose, 25mM Tris-HCl pH 8.0 and 5mg/ml lysozyme added prior to use.
Alkaline lysis solution II	200mM NaOH and 1%(w/v) SDS.
Alkaline lysis solution III	5M potassium acetate, pH4.8.
Chloroform:isoamylalcohol	This is a 24:1 mixture of chloroform and isoamylalcohol
Formamide dye mix	95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.
Gel soak I	600mM NaCl, 1M NaOH.
Gel soak II	600mM NaCl, 200mM Tris-HCl, adjusted to pH8.0 with HCl.
Hybridization buffer	7% SDS, 0.5M $NaPO_4$ (NaH_2PO_4; Na_2HPO_4),pH7.4.
Ligation buffer (10x)	200mM DTT, 100mM $MgCl_2$, 0.5M Tris-HCl, pH 7.8.
Phenol chloroform (1:1)	This is a 1:1 mixture of phenol and chloroform.
PBS -A	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH7.2.
PBS complete	PBS A plus 6.8mM $CaCl_2$, 4.9mM $MgCl_2$.
PBS /calf serum	PBS complete containing 5% newborn calf serum.
Random primer buffer (5x)	Three solutions A, B, C at a ratio of 10:25:15 respectively: solution A: 18µl 2-mercaptoethanol, 5µl 100mM dGTP, 5µl 100mM dATP, 5µl 100mM dTTP, 1ml (1.25M Tris-HCl, pH7.6, 0.125M $MgCl_2$). solution B: 2M HEPES, pH6.6. solution C: Hexadeoxyribonucleotides resuspended in TE at 90 OD units/ml.
RE stop	100mM EDTA, 10% (w/v) Ficoll 400, 0.25% (w/v) bromophenol blue, 5x TBE.
Saturated phenol	Phenol saturated by mixing 1:1 with phenol saturation buffer, (10mM Tris-HCl, pH7.5, 10mM EDTA, 100mM NaCl).

SSC (20x)	300mM trisodium citrate, 260mM NaCl.
TBE (1x)	2mM EDTA, 89mM boric acid, 89mM Tris-HCl pH 8.0.
TE	1mM EDTA, 10mM Tris-HCl pH8.0.
Tris-saline	140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml glucose, 0.0015% (w/v) phenol red, 25mM Tris-HCl (pH7.4), 100 units/ml penicillin, 0.1 mg/ml streptomycin.
Trypsin	0.25% (w/v) Difco trypsin dissolved in Tris-saline.
Trypsin-Versene	1 volume trypsin + 4 volumes versene.
Versene	0.6mM EDTA dissolved in PBS containing 0.002% (w/v) phenol red.
X-gal	25mg/ml or 100mg/ml in dimethylformamide.

2.1.15. Miscellaneous.

Hybond™ N hybridization transfer membranes	Amersham
Giemsa stain	BDH
Protein A-sepharose	SIGMA
Polaroid- 667 film	Polaroid (U.K.)
Repelcote	BDH Chemicals
XS-1 film	Kodak
EN^3-HANCE	Biotechnology Systems

2.2. METHODS.

2.2.1. Growth of cells

BHK21/C13 and HFL cells were grown in 80oz roller bottles containing 100ml ETC10 medium at 37°C for 3 days in an atmosphere of 95% air and 5% CO_2. Confluent cells were harvested by washing the monolayers twice with 25ml of trypsin-versene, and resuspending the detached cells in 20ml of ETC10.

The 3T6, NBL-6, RK13 and CV1 were grown in flasks and harvested under the same conditions using Dulbecco's Modified Eagle's medium. The yield of cells was determined as described by Freshney (1994) using an improved Neubauer counting chamber (Weber, England).

Cells were plated on 60mm or 33mm petri dishes or Linbro wells at a density of $4x10^6$, $2x10^6$ and $5x10^5$ cells per plate, respectively.

2.2.2. Preparation of virus stocks.

Virus stocks were prepared as described by Brown *et al.* (1973). Monolayers of NBL-6 cells in flasks were infected with 0.01 p.f.u./cell of EHV1, strain Ab4p in 20ml of Dulbecco's Modified Eagle's medium and incubated at 31°C for 3-4 days, until c.p.e. was complete, when the cells were shaken into the medium. The cells were pelleted in 250ml plastic falcon tubes by spinning at 2K for 10 minutes at 4°C in a Fison's Coolspin. The supernatant and cell pellet were separated and two individual virus stocks prepared:

Supernatant stock: The supernatant was poured into 250ml centrifuge bottles and spun at 12K for 2h (4°C) in a Sorvall GSA rotor. The supernatant was discarded, and the virus pellet resuspended in 1ml ETC10 or PBS/calf serum per flask. The pellet was sonicated until homogeneous, before aliquoting into 2ml amounts and storing at -70°C.

Cell-associated stock: The cell pellet was resuspended in 0.2ml medium/ flask and thoroughly sonicated before spinning at 2K for 10 minutes at 4°C in a Fison's Coolspin centrifuge. The supernatant was kept on ice while the process was repeated. The two supernatants were combined to give the cell-associated virus stock. This was aliquoted and stored as above.

In some cases the cell-associated and the supernatant stocks were mixed before aliquoting and storing at -70°C.

2.2.3. Sterility checks on virus stocks

Brain heart infusion agar (BHI) plates and BHI plates containing 10% horse blood (BHI blood agar) were used for checks.

To check for fungal contamination of stocks, a small aliquot was streaked onto BHI plates in duplicate, which were sealed with parafilm and incubated at RT. Bacterial contamination was detected by plating onto BHI blood agar and incubating at 37°C. If no colonies were visible

after 7 days incubation, the stocks were considered sterile.

2.2.4. Titration of virus stocks

Virus stocks were serially diluted 10-fold in PBS/calf serum. 100µl aliquots added to 75% confluent monolayers of BHK21/C13 cells on 60mm petri dishes from which the medium had been removed. The plates were incubated at 37°C for 1h, to allow adsorption of the virus to the cells. The plates were washed twice with PBS, before overlaying with 6ml EMC10%, and incubated at 37°C for 3 days. Monolayers were fixed and stained with Giemsa at RT for 3h. After washing, plaques were counted on a dissecting microscope and virus titres calculated as p.f.u./ml.

2.2.5. Preparation of EHV1 DNA

The DNA of EHV1 stocks was prepared according to the method of Wilkie (1973) and Brown *et al.* (1984). To prepare a large scale EHV1 DNA stock, 10 flasks containing almost confluent monolayers of NBL-6 cells were infected with virus at a m.o.i. of 0.01 p.f.u./cell. The infection was continued at 31°C until c.p.e. was extensive (3-4 days). The cells were shaken into the medium and spun at 2K for 10 minutes in a Fison's Coolspin. The supernatant was kept on ice while the nuclei were extracted from the cell pellet by treatment with 0.5% (w/v) NP40 in RSB (10mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl, pH 7.5) followed by centrifugation (2K for 10 minutes) to pellet the cell debris and nuclei. This was repeated before pooling the supernatants and spinning to pellet the virus at 12K for 2h in a Sorvall SS34 rotor.

The virus pellet, containing cell released and cytoplasmic virus was resuspended in NTE buffer (10mM Tris-HCl, pH7.5, 10mM NaCl, 1mM EDTA) before adding EDTA and SDS to a final concentration of 10mM and 2% (w/v) respectively to cause lysis of the virus. Viral DNA was extracted 3-4 times with saturated phenol and once with chloroform: isoamylalcohol, prior to precipitating with 2 volumes of ethanol at RT for 5 minutes. DNA was pelleted at 2K for 10 minutes, washed with 70% ethanol, dried at 37°C and resuspended in a minimal volume of H_2O containing RNase A (50 µg/ml).

2. 2. 6. Transfection of DNA into cells using the calcium phosphate technique.

This method is a modification of that described by Stow and Wilkie (1976). Four hundred µl HEBS (130 mM NaCl, 4.9 mM KCl, 1.6 mM Na_2HPO_4, 5.5 mM D-glucose, 21 mM HEPES, pH7.05) containing 10 µg/ml calf thymus DNA, 1-2 µg intact virus DNA, 1-10 µg plasmid DNA and 130 mM $CaCl_2$, were added to confluent monolayers of BHK21/C13 cells on 60mm petri dishes, from which the medium had been removed. Following incubation at 37°C for 40 minutes, cells were overlaid with 6ml ETC10.

Four to eight hour later the media was removed from the plates and they were washed twice with ETC10. 1.5ml 25% (v/v) DMSO in HEBS was added and the plates incubated at RT for 4 minutes. The DMSO was removed and the plates washed twice and overlaid with 6ml ETC10.

Incubation was continued at 37°C until c.p.e. was complete.

2. 2. 7. Isolation of single plaques from transfection experiments.

Once c.p.e. was complete, the cells were scraped into the growth media, transferred into a small glass vial and sonicated until homogeneous. Serial 10-fold dilutions of each transfection were made in PBS/calf serum and the 10^{-3} to 10^{-7} dilutions from each plated onto 70% confluent BHK21/C13 monolayers. After adsorption at 37°C for 1h, the plates were overlaid with 4ml EMC10% and incubated at 37°C for 30 h. 2 ml EMC10 containing 0.6 mg/ml of X-gal was added to the plates and the plates were incubated for a further 12 h at 37°C. The plates were washed twice and single blue plaques were picked and seeded into linbro wells containing monolayers of BHK21/C13 cells. The cells were incubated for 2-3 days at 37°C. When c.p.e. was complete, the linbro wells were stored at -70°C for temporary stock.

2. 2. 8. Marker rescue

In vitro marker rescue methods were used to restore the deleted gene phenotype of the mutant viruses. DNA (1µg) from a mutant virus was transfected onto BHK21/C13 cells with a 1-, 10- and 100- fold molar excess of a wild-type fragment containing the deleted gene ORF. The following day, X-gal (0.6 mg/ml in EMC10) was added into the medium. After two days, white plaques were picked and their genomic structures were identified by restriction enzyme digestions and Southern blotting. Before stock virus was prepared, the revertant was single plaque purifed four times.

2. 2. 9. *In vivo* preparation of ^{32}P labelled viral DNA

This is a modification of the method described by Lonsdale (1979). Confluent monolayers of BHK21/C13 cells in Linbro wells were infected with either 100µl of a plate stock or $2x10^6$ p.f.u. of a virus stock. After adsorption for 45 minutes at 37°C, the virus was removed and the cells washed with and maintained in 450 µl PIC for 2h at 31°C. One µCi of orthophosphate (^{32}P) in 50 µl PIC was added per well and incubation continued at 31°C for 2-3 days. At the end of the incubation period, 0.5ml of 5% (w/v) SDS was added to each well. Trays were incubated at 37°C for 5-10 minutes before scraping the cells off the wells using a blue tip and adding them to 1ml of phenol. The samples were inverted and incubated at RT for 10 minutes, with a further inversion after 5 minutes. They were centrifuged at 2K for 10 minutes at RT in a Fison's Coolspin before removing the top layer into 2 volumes of ethanol. After gently inverting the tubes, they were spun as before. The ethanol was poured off and the DNA dried for 10 minutes at 37°C with the test-tubes in an inverted position. Two hundred µl of RNAse A (50 µg/ml) solution was added to each sample and left to dissolve for 2h at 37°C. The DNA was then ready for digestion with the appropriate enzyme(s).

2. 2. 10. Preparation of small amounts of virus infected cell DNA for Southern blotting

BHK21/C13 cell monolayers in 24 well linbro tray were infected at a m.o.i. of 5 pfu/cell at 37°C. After 48 h the supernatant was removed and the cells lysed by incubating with 2ml of lysis buffer (0.6% (w/v) SDS, 10mM EDTA, 10mM Tris-HCl pH 74) containing 500µg/ml protease, for 4 h at 37°C. Infected cell DNA was extracted twice with an equal volume of phenol and once with chloroform; precipitated by the addition of 2 volumes of ethanol and dried in a vacuum desiccator. The DNA was dissolved in 200µl of water containing 50µg/ml RNase A. Restriction enzyme digests were usually carried out on 5% of the total sample (10µl).

2. 2. 11. Purification of EHV1 virions.

Virions were purified essentially by the method of Szilagyi & Cunningham (1991). Monolayers of BHK21 C13 cells in roller bottles were infected with EHV1 strain Ab4 at a m.o.i. of 0.01 pfu/cell and incubated at 31°C for 3 days. Once c.p.e. was complete the supernatant was harvested and pelleted by low speed centrifugation (1,000g for 10 min at 4°C), and the virus particles in the clarified medium were pelleted by centrifugation at 12,000 r.p.m. for 2 h at 4°C. The pellet was then gently resuspended in 1 ml of modified medium (culture medium without phenol red or calf serum) and layered onto a 35-ml performed gradient of 5 to 15% (w/v) Ficoll 400 (Sigma) suspended in this medium. After centrifugation in a swing-out rotor (12,000 r.p.m. for 2 h at 4°C in an AH629 cellulose nitrate tube), the higher L-particle band and lower virion band were withdrawn by side puncture. These virions were diluted in modified medium and pelleted by centrifugation (21,000 r.p.m. for 2 h at 4°C in an AH629 tube). The virion pellet was gently resuspended in 200µl of modified medium and either used immediately or stored at -70°C.

2. 2. 12. Analysis of virus growth properties *in vitro* .

Virus growth experiments *in vitro* were carried out essentially as described by Brown *et al* (1994). For multi-cycle growth experiments, confluent cell monolayers were infected at a multiplicity of 0.01 p.f.u./cell of stock viruses. Single-cycle growth experiments were carried out by infecting confluent monolayers at a multiplicity of 5 p.f.u./cell. After 1 h absorption, the monolayers were washed three times with PBS and overlaid with ETC10. At specific times post-infection, cells were harvested, sonicated and stored at -70°C. Viruses were titrated on monolayers of BHK21/C13 cells at 37°C.

2. 2. 13. Temperature sensitivity

Confluent BHK21/C13 cells were infected in triplicate with stock viruses at a multiplicity of 5 p.f.u./cell. After 24h incubation at 31°C, 37°C, and 38.5°C, the monolayers were harvested, sonicated and the progeny virus titrated on BHK21/C13 cells at 37°C.

2.2.14. Assay for rate of virus adsorption

Monolayers of BHK21/C13 cells ($4x10^6$) in 50 mm petri dishes were precooled for 1 hr at 4°C and inoculated with virus at a multiplicity of infection (m.o.i.) of 300 pfu/plate and left at 4°C for various lengths of time up to 4 hr. At various time points, the plates were washed three times with PBS and overlaid with medium containing 1% carboxyl methyl cellulose. The plates were incubated at 37°C for 2 days after which time they were fixed and stained. Plaques were counted, and the % of virus absorbed at each time-point determined, relative to the final time-point (4 hr) which represented 100% adsorption.

2 2.2.15. Assay for rate of virus penetration

Virus penetration was assessed by determining the rate at which adsorbed virus became resistant to inactivation by a low-pH citrate buffer. The method used was essentially as described by Huang and Wagner, (1964) and Highlander *et al.*, (1987). Confluent BHK21/C13 cells ($4x10^6$) in 50 mm petri dishes were separately infected with 400 pfu per plate of either purified wild-type virus, the mutant ED71 or Re71 and allowed to adsorb for 2h at 4°C. The unbound virus was removed by washing four times with PBS. The cells were then overlaid with Eagle's medium and shifted to 37°C to allow viral penetration to proceed. At selected time points after the temperature shift, plates were either treated with 1 ml of citrate buffer (40 mM citric acid, 10mM KCl, 135mM NaCl, pH 3.0) or with 1 ml of PBS for 5 min as a control. The monolayers then were washed three times with PBS and overlaid with Eagle's medium containing 1% carboxyl methylcellulose and incubated at 37°C for 2 days. The plaques were visualised with Giemsa stain and counted. Experiments were performed in triplicate. The amount of penetrated virions at each time point was determined as % of pfu from citrate buffer treated plate composed to PBS control.

2.2.16. Assay for virion release from infected cells.

BHK21 C13 monolayers in 33 mm petri dishes were separately infected with ED71, wild-type virus or Re71 at a multiplicity of infection of 5 pfu/cell. After 1 h adsorption at 37°C, the unbound extracellular virus was removed by thoroughly washing the monolayers four times with PBS containing 1% calf serum. After each incubation period, the medium was removed, clarified at 1500 rpm for 5 min at 4°C and stored at -70°C. Cells were separately harvested in the same volume, sonicated and stored at -70°C until the titres were assayed.

2.2.17. Assay for virus spread from cell to cell.

Confluent monolayers ($4x10^6$) of BHK21/C13 cells were infected with virus at a m.o.i. of 0.01 pfu/cell. After adsorption at 37°C for 1.5 h, the plates were washed twice with PBS, overlaid with ETC10 plus and minus, EHV1 neutralising horse serum (final concentration 1:25) and incubation continued at 37°C. The infected cells were harvested at various time points after

infection and virus titrated on BHK21/C13 cells.

2.2.18. Electron microscope analysis

BHK21/C13 monolayers (4 x 10^6 cells) infected with virus at 3 pfu/cell, were scraped from petri dishes at 24 h post infection and pelleted in Been Capsules (Taab Laboratories). The pellet was fixed in 2.5% (v/v) glutaraldehyde in PBS, then post fixed in 1% (aqueous) OsO_4. The pellets were dehydrated through a series of increasing ethanol concentrations to 100%, then infiltrated with Epon 812 epoxy resin (Taab Laboratories). After polymerisation 80 nm sections were cut and stained with saturated uranyl acetate (in 50:50 ethanol/water) and subsequently stained with lead citrate. For counting, 50 complete cell profiles (non-serial and including nuclei) were examined.

2.2.19. Preparation of infected cell extracts

Infected cells were washed once with PBS and incubated with either extraction buffer [100 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 0.5% (v/v) NP40, 0.5% (w/v) Na deoxycholate, 500uM PMSF (phenylmethylsulphony fluoride)] for immunoprecipitation. For Western blotting samples were harvested with sample buffer (50mM Tris pH 7.5, 2% (v/v) SDS, 7mM 2-mercaptoethanol, 10% (v/v) glycerol) at 0°C for 30 min. The cells were scraped into buffer and then spun in a Beckman microfuge for 10 min and the supernatant stored at -70°C until use.

2. 2. 20. Radiolabelling of infected cell extracts

Monolayers of BHK21/C13 cells in 33 mm Petri dishes were infected with virus at a m.o.i. of 5 pfu per cell. Virus was allowed to adsorb for 1 h at 37°C and the plates were washed twice with PBS and further incubated in 2ml ETC10 medium. If labelled extracts were required, 50 µCi/ml of [^{35}S]-methionine in Emet/5C2 , 100 µCi/ml of [^{32}P]-phosphate in PIC or either 2.5 µCi/ml of [^{14}C]-glucosamine or 3 µCi/ml of [^{3}H]-mannose in Eglu/10C2, were added to the cells and incubated usually from 5 h to 20 h post-infection (pi) (0 h pi being taken as the end of the 1 h absorption period). At 24 h p.i. the cells were washed once with PBS and scraped into either extract buffer (section 2.2.26.) for immunoprecipitation or sample buffer (section 2.2.27) for Western blotting.

2. 2. 21. Surface labelling of virion glycoproteins

Exposed carbohydrate residues of viral glycoproteins on the virion envelope surface were labelled *in vitro* by the enzymatic labelling method of Wallenfels (1979). Briefly, the reaction mixture containing (in a total volume of 460µl) 0.25 M Tris-HCl pH7.4, 400µg purified virions, 2µCi UDP-[^{14}C] galactose (270mCi/mmol), 0.28 units of galactosyl transferase and 0.02 M $MnCl_2$ was incubated with shaking for 1 h at 37°C, followed by 5 min at 0°C. The labelled virions were washed by centrifugation in 10mM-Tris pH 7.5 and 1mM-EDTA to remove unincorporated UDP-[^{14}C] galactose. The pellet was then resuspended in sample

buffer (2.2.7. section) and analysed by SDS-PAGE.

2. 2 22. Preparation of virus-induced early polypeptides

To prepare extracts of proteins produced in the absence of viral DNA replication (loosely termed 'early' extracts) cell monolayers were pre-treated with phosphonoacetic acid (PAA) at 300 µg/ml for 1h, then infected and maintained in the continuous presence of PAA. Cells were harvested with sample buffer at various times p.i.

2. 2. 23. Preparation of virus-induced immediate early polypeptides

BHK21/C13 monolayers in 33-mm petri dishes were preincubated for 1.5 h before virus infection in ETC10 containing 200 µg/ml of cycloheximide. Cells were infected at a multiplicity of infection of 10 pfu per cell; after 1 h adsorption, cells were washed and incubated for a further 5 h, all in the same medium. Actinomycin D was then added to a final concentration of 2.5 µg/ml. After 15 min, this cycloheximide-containing medium was removed, and plates were washed twice with medium containing 2% calf serum and actinomycin D (2.5 µg/ml) and incubated for a further 2 h. Control cultures were treated in the same manner except that cycloheximide was omitted. The cells were washed once with PBS and lysed with sample buffer and analysed by Western blotting.

2. 2.24. Subcellular fractionation

Subcellular fractionation was based on the method described by Bryant & Ratner (1990) and MacLean *et. al.* (1992) with some modification. Cell monolayers were rinsed twice with PBS and once with distilled water before being scraped into homogenization buffer (20 mMTris-HCl pH8, 10 mM $MgCl_2$, 0.5 mM EDTA, 7 mM 2-mercaptoethanol) and swollen on ice for 20 min. The cells were then disrupted by Dounce homogenization (50 strokes) and centrifuged at 2000 r.p.m. for 3 min at 4 °C in a Fison's Coolspin to remove nuclei and any intact cells. The supernatant material was adjusted to 150 mM-NaCl and centrifuged in a Sorvall Ti50 rotor at 45000 r.p.m. for 30 min at 4°C. The supernatant (cytosol) was removed and stored at -70°C, and the pellet (total membranes) was resuspended in homogenization buffer containing 150mM-NaCl. A sample of the total membrane fraction was then adjusted to 1M-NaCl and 100mM-sodium carbonate buffer (pH 11.5) to bring the protein concentration to 1mg/ml and incubated at 4°C for 30 min, and centrifuged as before. The supernatant contained those proteins which bound only at the low ionic strength, whereas the pelleted materials contained the proteins which bound to membranes at high ionic strength. The pellet containing high affinity membrane bound proteins was gently washed once with ice-cold distilled water and dissolved in 300µl of 1x sample buffer (Section 2.2.21.), before storing at -70°C.

2. 2. 25. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Vertical slab gels were used for protein analysis. Fresh stocks of 30% (w/v) acrylamide were

prepared in water at a ratio of acry-lamide:n,n'-methylene bisacrylamide (the cross-linking agent) 29.25:0.75. If the gels were cross-linked with N,N'-diallyltardiamide (DATD) the stocks of 30% (w/v) acrylamide were prepared in water at a ratio of acrylamide:DATD 92.25:0.75. These were then filtered through Whatman N°1 filter paper before use. From these stocks, gel solutions containing the appropriate percentage of acrylamide were prepared in a buffer of final concentration 375mM Tris-HCl, pH8.9, 0.1% (w/v) Sodium dodecyl sulphate (SDS). To polymerise the gels, ammonium persulphate and TEMED were added at a final concentration of 3.65 mM and 2.75 mM, respectively. The gel solutions were poured between two well-washed glass plates separated by 1.5 mm spacers to within 3-4 cm of the top of the glass-plate sandwich. 400 µl of butan-2-ol was loaded on the top of the acrylamide solution, and following polymerisation a stacking gel, consisting of 5% (w/v) acrylamide in 122 mM Tris-HCl pH 6.7, 0.1% (w/v) SDS, was added, into which a twenty-one well-forming comb was placed.

2. 2.26. Immunoprecipitation

100-200µl of infected or mock infected cell extracts were incubated with rabbit antiserum (5-10µl) for 16 h at 4°C. Following incubation, 60µl of protein A-sepharose (Sigma), diluted 1:1 in the extraction buffer, was added and incubation continued at 4°C for a further 1-2 h. The protein A-sepharose pellet was then washed 4 times with the extraction buffer and the bound polypeptides on the protein A sepharose eluted by boiling the beads for 5 min in sample buffer and analysed by SDS-PAGE. The gels were fixed in 6.1% acetic acid and 46.7% methanol and soaked in EN^{3}HANCE for 1h and washed in H_2O for 20 min. The gels were dried and exposed to Kodak X-Omat films for fluorography.

2. 2. 27. Western blotting

Infected cells were harvested into sample buffer at a concentration of 10^7 cells/ml. After boiling for 5 min, samples were loaded onto SDS-PAGE at approximately 10^6 cells /well. Following electrophoresis, the gel was placed on a sheet of Whatman grade 182 filter paper presoaked in transfer buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 20% (v/v) methanol) on top of a foam pad and a pre-soaked nitrocellulose membrane was placed face down on the gel, with care being taken to exclude air bubbles between the gel and the membrane. Finally a further sheet of pre-soaked filter paper and then another foam pad were placed over the gel and the sandwich (held tightly together in a plastic holder) was placed in an electrophoresis tank with the gel towards the cathode and the nitrocellulose towards the anode. Proteins were transferred to the nitrocellulose by electrophoresis in transfer buffer for at least 3 h at room temperature. The nitrocellulose sheet was blocked with 2% dried milk in PBS/0.05% (v/v) Tween 20 for 1 h at 37°C and incubated with 1:25 - 1:200 diluted antiserum in PBS/Tween 20 0.05% (v/v) for 16 h at 4°C. The sheets were washed in PBS/Tween 20 0.05% (v/v) by gently shaking at 37°C, with 1 change of buffer over 15 min. The sheets were incubated with 1:1000

diluted horseradish peroxidase(HRP)-labelled protein-A conjugate in PBS/Tween 20 containing 1% (w/v) BSA. The sheets were then washed extensively in PBS/Tween20 with at least 3 changes over 1 h at 37°C. Finally bound antibodies on the sheets were detected with the ECL system (Amersham). The sheet was incubated with equal volumes of detection reagent 1 and 2 for 1 min at room temperature. Following incubation, the detection reagents were drained off and the sheets were wrapped in Saran Wrap and exposed to Kodak film for 10 to 60 seconds.

2. 2. 28. Immunofluorescence staining.

At 16 h p.i. or 30 h post transfection, cells on glass coverslips were washed once with PBS and fixed with 4% formaldehyde in PBS, pH7.4 for 10 min at room temperature; washed three times and permeabilised by 0.2% (v/v) Triton-X 100 in PBS for 30 min at room temperature. The cells were washed and incubated with a specific rabbit antiserum (dilution of 1:100) for 1h at 37°C. The cells were washed three times with PBS and incubated with the second anti-rabbit IgG (whole molecule) Fluorescien Isothiocyanate (FITC) conjugated-antibody (1:80 dilution) at 37°C for 1h. If the first antibody was a monoclonal antibody, an anti-mouse IgG TRITC conjugated-antibody was used as the second antibody. The cells were then washed four times with PBS, before removing the coverslips, polishing the underside and mounting them on microscope slides using a small amount of fluorocolor. The coverslips were sealed with clear nail polish and immediately examined or stored in the dark at 4°C for up to a few weeks .

2. 2. 29. NP40 treatment of purified virions.

The method used is described by Frame et al. (1987). Briefly, purified virions were incubated in the presence of 5% (v/v) NP40 and 600 mM-NaCl for 30 min at room temperature, and then diluted fivefold with 20 mM-tris HCl pH 7.5, and centrifuged in a Sorvall SS34 rotor at 12000 r.p.m. for 2 h at 4°C. One third volume of sample buffer was added to the supernatant. The non-soluble pellet was resuspended in 1x sample buffer in a equivalent volume to the supernatant fraction. The samples were used immediately or stored at -70°C.

2. 2. 30. Digestion of EHV1 virion glycoproteins with glycosidases

(1) Purified EHV1 virions (600µg) were incubated with 10 µl of 1% (v/v) Triton X-100 in PBS at 0°C for 10 min and centrifuged at 10,000 g for 10 min. The supernatant was digested with 10 units/ml N-glycosidase [peptide-N-4(N-acetlyl-b-glucosaminyl) asparagine amidase, Genzyme] in 15µl of 0.2M-sodium phosphate buffer pH 8.6 and 3µl of 100mM-1,10-phenanthroline at 37°C for 6 h.

(2) Following the treatment with N-glycosidase, one third of the above digestion half stored at -70°C. The remaining two thirds was adjusted to pH5.0 with 0.1M-citric acid and digested

with 0.2 units of neuraminidase(acetylneuraminyl hydrolase, Sigma) at 37°C for 4 h.

(3). Finally, half of the above digestion was stored at -70°C and the remaining was digested again with 0.01 units of ß-N-acetylhexosaminidase, 0.01 units of L-fucoside fusohydrolase, Sigma and 0.01 units of ß-galactosidase(ß-D-galactosidase galactohydrolase, Boehringer Mannheim) at 25°C for 4 h. The digested virions were boiled with 1x sample buffer (2.2.7 section) for 5 min and subjected to Western blotting.

2. 2. 31.Treatment of infected cells with inhibitors.

Confluent BHK21/C13 cell monolayers were infected with virus at a multiplicity of 20 pfu/cell or mock-infected. The cells were then incubated with medium in the presence of either 2 µg/ml tunicamycin (Sigma), 1 µM-monensin (Sigma), 40 µM Nocodazole(Sigma) or 40 µM Demecolcine (Sigma) at 37°C from the end of the adsorption period (1h) to 20 h p.i, respectively. The cells were then subjected to either Western blotting or immunofluorescence.

2. 2. 32. Lipofection of BHK21/C13 cells with plasmid DNA

To transiently express the gene 67 protein product, BHK21/C13 cells were lipofected with pCMV67 DNA according to the method of Felgner *et al.*, (1987). Briefly, 50% confluent monolayers of BHK21/C13 cells in 33 mm petri dishes were incubated with 2 µg of either pCMV67 DNA or pCMV10 DNA and 15µl of liposomes in 1ml Opti-MEM 1 (GIBCO BRL) at 37°C for 4 h. The medium was then replaced by ETC5 and the incubation continued at 37°C for a further 26 h. The cells were then either fixed for immunofluorescence or lysed for Western blotting.

2. 2. 33. Ligation.

Several strategies of ligation (Sambrook *et al* , 1989) were adopted to ligate fragments of EHV1 DNA to plasmid vectors. When both vector and DNA fragments cut with the appropriate restriction enzymes (RE) produced compatible or blundends of fragments, various quantities of fragment were ligated to 100ng of vector DNA in 1x ligation buffer (see Section 2.1.14) with 2 units of T4 DNA ligase at 16°C overnight. If the vector and fragment were cut with inappropriate REs, two types of fragment ends (5' overhangs and 3'overhangs) could be generated. A fragment with 5' overhangs had to be filled in to produce a blunt end fragment with 0.1 volume of 2.5mM dNTPs and 1 unit of DNA polymerase I, which carries the 5´-->3´ DNA polymerase activity, was added to the digestion mixture; if the fragment had 3' overhangs, they had to be exonucleased into blunt ends with 1 unit of T4 DNA polymerase, which has a strong 3'-->5´ exonuclease activity in 1x T4 polymerase buffer before cloning into the vector cut with a blunt-cutting enzyme e.g. Sma I.

2. 2. 34. Glycerol stocks of bacteria.

Glycerol stocks of bacteria were prepared by centrifugation of an overnight culture (10ml) at 2000 rpm for 10 min and resuspending the pellet in 5ml L-broth, mixed with 5ml of 99.5% glycerol and storing at -20°C.

2. 2. 35. Preparation of competent bacteria and transformation

Ten ml of 2xYT broth was inoculated with 15µl of a glycerol stock of bacteria and incubated with shaking at 37°C overnight to produce a saturated culture. One ml of this culture was used to inoculate 100ml of 2xYT broth which was shaken at 37°C for 2.5-3h to get the bacterial cells into mid-log phase. The bacterial cells were pelleted by spinning at 2K for 10 minutes in a Fison's Coolspin and resuspended in 1/10th volume of transformation and storage buffer (10 mM $MgCl_2$, 10mM $MgSO_4$, 10%(w/v) PEG 3,500, 5% (v/v) dimethyl sulphoxide). After 15 minutes on ice, the bacteria were considered competent for transformation. Typically, 5µl of a ligation mix or 1 µl plasmid preparation were incubated for 30 minutes on ice with 100 µl of competent *E.coli.* One ml of L-broth was added to the transformation mix and incubation was continued for a further hour at 37°C. For selection of transformations, 100µl of the transformed bacteria were plated on to L-broth agar plates containing appropriate antibiotics, such as 100 µg/ml ampicillin and ± 25µg/ml X-gal. Plates were allowed to dry at room temperature, before incubating in an inverted position at 37°C overnight (Chung and Miller, 1988).

2. 2. 36. Small scale plasmid preparation.

Single, transformed, bacterial colonies were picked and resuspended in 5ml of L-broth containing 100µg/ml ampicillin. Following growth at 37°C for 8-16h, 1.5ml aliquots of each culture were spun at low speed (6500g) in a microfuge, the supernatant discarded and the cells resuspended in 100µl of alkaline lysis solution I (section 2.1.14). Following 5 minutes incubation at RT, 200µl of alkaline lysis solution II (section 2.1.14) was added, and the cultures mixed vigorously. After a further 5 minutes incubation at RT, 150µl of alkaline lysis solution III (section 2. 1.14) was added, mixed by vortexing and incubated at RT for 5 minutes. A white precipitate consisting of the cell debris was pelleted at high speed (13000g) in a microfuge. Plasmid DNA was extracted from the supernatant using an equal volume of phenol:chloroform followed by 2 volumes ethanol precipitation at RT for 1 minute. The DNA was pelleted by spinning at 13,000g for 5 minutes in a microfuge, washed in 70% ethanol, dried in a Speedivac and resuspended in 100µl H_2O containing 50 µg/ml RNase A (Sambrook, *at al.*, 1989).

2. 2. 37. Large scale plasmid preparation.

The method used was essentially as described by Maniatis *et al* ., (1982). A single transformed bacterial colony from a L-broth agar plate was inoculated into 5ml of L-broth containing the

appropriate antibiotic and shaken at 37°C for 8-16h. The culture was transferred into 500ml L-broth containing the appropriate antibiotics in a 2 litre dimpled flask and shaken at 37°C overnight. The bacteria were pelleted by centrifugation at 8K for 10 minutes in a Sorvall GSA rotor, the pellet resuspended in 10ml alkaline lysis solution I (see section 2.1.14.) and incubated at RT for 10 minutes. Freshly made alkaline lysis solution II (20ml) was added and incubation continued for a further 10 minutes on ice. Ice-cold alkaline lysis solution III (15ml) was added, incubation continued on ice for 10 minutes and the bacterial debris pelleted by centrifugation at 12K for 10 minutes in a Sorvall SS34 rotor. DNA was extracted twice with an equal volume of phenol and once with an equal volume of chloroform. The DNA was precipitated by the addition of 2 volumes of ethanol, centrifuged at 2K for 30 minutes in a Sorvall SS34 rotor at RT, washed in 70% ethanol, pelleted as before, dried in the incubator and dissolved in water containing 50 µg/ml RNase A.

In some instances, to remove residual host DNA and RNA, the DNA was further purified by isopycnic banding on ceasium chloride gradients. In this case, the DNA was only extracted once with phenol:chloroform. Ceasium chloride was added until the buoyant density was 1.55g/ml and ethidium bromide was added to a final concentration of 0.5 mg/ml. The mixture was pipetted into Oakridge tubes, which were capped, sealed and centrifuged at 45,000 r.p.m. overnight in a Sorvall Ti50 rotor. The DNA was visualised on a long wave U.V. light box and the lower band which contained the supercoiled plasmid DNA was removed using a needle and syringe. After extracting the ethidium bromide at least 3 times with isoamyl alcohol, the DNA was ethanol precipitated as described above. The DNA concentration was quantitated by running a small quantity on an agarose gel alongside standards of known concentrations (Sambrook *et al* .,1989).

2. 2. 38. Restriction enzyme digestion of DNA

The manufacturer's recommended buffers and conditions were generally used for each individual restriction enzyme. To achieve complete digestion, 1µg samples of EHV1 or plasmid DNA were digested with 2-5 units of restriction enzyme for 4h at the appropriate temperature. If the digested DNA was to be run on an agarose gel, 1/5- 1/6 volume of RE stop was added prior to loading, otherwise the digested DNA was recovered as described in Section 2.2.41.

2. 2. 39. Phenol-chloroform extraction of DNA from restriction enzyme digestion mixtures.

If the digestion mixture was not at least 100µl, it was increased to this volume using dH_2O. An equal volume of phenol:chloroform was added, the mixture vortexed vigorously, and spun at 13000g for 2 minutes in a microfuge. The top aqueous layer was removed into a separate eppendorf tube and the bottom layer was back extracted with an equal volume of dH_2O. An

equal volume of chloroform was added to the aqueous layer and the mixture mixed and spun at 13000g for 2 minutes in a microfuge. The top layer was removed and the sample back extracted as before. To precipitate the DNA, 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate were added. Following 15 minutes incubation on dry ice, the DNA was pelleted at 13000g for 5 minutes, washed with 70% ethanol and dried in a Speedivac, before resuspending in an appropriate volume of TE.

2. 2. 40. Agarose gel electrophoresis.

Restriction endonuclease analysis of viral or plasmid DNA used 0.4-2% (w/v) agarose gels boiled in 1xTBE buffer. When cooled to about 45°C, the solutions were poured on to glass plates (16.5x26.5cm) whose edges had been sealed with gel tape and on to which 12-26 tooth combs had been placed. For non-radioactive samples, ethidium bromide was added to the agarose solution to a final concentration of 0.5mg/ml before pouring. The gels were allowed to set at RT for 1h and then transferred to horizontal tanks containing 1xTBE buffer. DNA samples were mixed with one-fifth volume of RE stop, loaded into the gel tracks and electrophoresed at 40-120V for 4-16 h (Sambrook *et al*., 1989).

For quantitation of fragment and vector DNA prior to transfection or ligation, gels containing ethidium bromide were poured in to a mini-gel Kit (Bio-Rad). Samples (generally 5-7µl) were run at 50 V for 1-2h, visualized using a short wave (260) U.V. lamp and photographed on Polaroid film. A 1Kb DNA marker (BRL) of known concentration was run alongside the DNA samples to enable confirmation of the vector/fragment size and quantitation of each sample.

2.2.41. Recovery and purification of DNA from agarose gels .

Generally the DNA to be recovered had been digested with restriction enzymes before running on an agarose gel. In order to cause the minimum possible damage to the DNA, it was visualised using a long wave (300-360nm) U.V. lamp, a gel slice containing the desired DNA fragment cut out with a sharp scalpel and the DNA recovered and purified using Prep-A-Gene DNA Purification Kit (BIO-RAD) in accordance with the manufacturer's instructions. For this purpose, the gel slice was cut into small pieces and dissolved at 55°C in the presence of Prep-A-gene binding buffer. The proper amount of Prep-A-gene matrix (20µgDNA/25µl) was added, the mixture vortexed briefly, incubated at RT for 10 min and spun at 2000rpm for 2 min. The pellet containing the bound DNA was washed twice with the binding buffer and three times with the wash buffer. After the last wash, the bound DNA was eluted at 37°C using elution buffer. The yield was estimated as described in Section 2.2.40.

2. 2. 42. Southern blotting.

Southern blotting was carried out as described by Sambrook *et al.* (1989). Purified virus, infected cell DNA or plasmid DNA was digested with the appropriate restriction enzyme(s) before running on an agarose gel. The gel was visualised under short-wave U.V. light to

confirm DNA digestion, then placed in 500ml Gel Soak I for 1h, rinsed with deionised water and transferred to Gel Soak II for 1h. After rinsing as before, it was transferred to 500ml 20xSSC for a further 1h. The gel was now ready for blotting onto either one sheet or two sheets simultaneously of Hybond N hybridization transfer membrane. For each transfer one sheet of membrane and five sheets of Whatmann 3MM chromatography paper, all cut to the exact size of the gel were required.

A bundle of 'Hi-Dry' towels was placed on the bench followed by three sheets of dry then two sheets of 20xSSC soaked 3MM paper. The membrane was placed on top of the filter papers followed by the gel, ensuring that there were no air bubbles between the gel and the membrane. A glass plate and heavy weight were placed on top and left for at least 4h during which time the DNA was drawn out of the gel and onto the adjacent side of the nitrocellulose. To transfer DNA to two membranes, this procedure was repeated on top of the gel.

The DNA was cross-linked to the membrane using a U.V. Stratalinker (Stratagene, USA) and hybridized to the random produced probe in a sealed bag containing 20ml hybridization buffer (see section 2.1.14). Hybridization was generally carried out overnight at 65°C. The membrane was washed for 3x30 min with 1 litre 2xSSC, 0.1%(w/v)SDS before drying and setting up for autoradiography against Kodak XS-1 film.

2. 2. 43. Purification of synthetic oligonucleotides

Eighty ml of 6% (w/v) acrylamide, 1xTBE, 9M Urea sequencing gel mix was polymerized with 160µl of 25% (w/v) APS and 60µl TEMED. This was then poured between two 20x22cm glass plates separated by 1.5mm spacers and a 10 tooth comb inserted at the top. The synthetic oligonucleotides were eluted from the column with ammonium for 2 h and deprotected at 55°C for 5-6h, frozen on dry ice and dried in the Speedivac overnight. The oligonucleotide was resuspended in 100µl H_2O. Glycerol (5µl) was added to 30µl of oligonucleotide solution and loaded immediately onto the gel. Formamide dye mix (2µl) was loaded in a separate well to act as a molecular weight marker and the gel was run at 10mA for 3-4h in 1xTBE. The electrophoresis was stopped when the dye had migrated approximately two thirds of the length of the gel. The gel was removed, wrapped in cling-film and viewed against a white chromatographic plate using a hand-held long-wave U.V lamp (Sambrook *et al.,* 1989). If the synthesis had been successful, then a predominant band with possibly a few lower molecular weight bands was observed. The top band was cut out with a scalpel, mashed with a glass rod, and incubated overnight at 42°C in 1ml elution buffer (0.5M ammonium acetate, 0.25mM EDTA, 20% (w/v) SDS). This was filtered through a SPIN-X centrifuge unit (Costar) to remove the acrylamide, phenol: chloroform extracted, ethanol precipitated, washed in 70% ethanol, dried and redissolved in dH_2O. To quantitate the DNA, the OD_{260} was read and the conversion factor: 1 OD unit=20µg/ml was used.

2.2.44. Sequence analysis of doublestrainedplasmid DNA.

Sequence analysis was carried out using the Sequenase Version 2.0 kit (Amersham) in accordance with the manufacturer's instructions. Plasmid DNA (20µg) was denatured and annealed to 30 ng of the 15-mer oligo primer which hybridizes upstream of the multicloning site of the pUR vector in a 5x annealing buffer (200mM Tris-HCl , pH7.5, 100mM $MgCl_2$ and 250mM NaCl) in a total volume of 10µl. Annealing was carried out by first heating to 55°C for 5 minutes in a 1.5ml eppendorf tube, then allowing to cool to room temperature over a period of 30 minutes. The templates were briefly chilled on ice, before being labelled using a buffer which contained 1.5mM dCTP, 1.5mM dGTP, 1.5mM dTTP, 5µCi ^{35}S dATP, 6mM DTT and 3 units of Sequenase T7 DNA polymerase in a total volume of 16µl. Labelling was carried out at RT for 5 minutes. Each template was extended and terminated by addition of 3.5µl of labelled template to 2.5µl of each of the 4 ddNTP termination mixes in microtitre wells, which had been pre-warmed to 37°C. Each ddNTP termination mix contained one ddNTP at a concentration of 8mM and dNTPs at a concentration of 80 mM. The plate contents were mixed by centrifugation in a Beckman centrifuge and the reactions carried out at 37°C for 10-30 minutes. Four µl formamide dye mix was added to each sample. The samples were boiled for 2 minutes before loading onto a gel for electrophoresis.

2 .2.45. Electrophoresis and autoradiography of sequencing gels.

Electrophoresis was carried out at 70W through vertical gels 42x34x0.04 cm in size. Gels consisted of 6% (w/v) acrylamide (cross-linked with 5%(w/v) N,N'-methylbisacrylamide) and 9M urea in 1xTBE. Polymerization was achieved by addition of 0.05%(w/v) APS and 0.1%(v/v) TEMED. Spacers and gel combs were supplied by Gibco-BRL. Both plates were treated with repelcote enabling the gel to be transferred to Whatmann 3MM chromatography paper following electrophoresis and dried under vacuum. Dried gels were then exposed to XS-1 film (35x43 cm) and developed using a X-omat processor.

2. 2. 46. Expression and purification of fusion proteins in *E. coli* .

A 5 ml overnight culture of *E. coli* transformed with the recombinant plasmid was added into 100 ml L-broth containing 100µg ampicillin. The culture was incubated with shaking at 37°C for 2 h, then IPTG (1µM) added and incubation continued for another 2 h at 37°C. Inclusion bodies were purified as described by Harlow and Lane, (1988). Cells from the 100 ml culture were pelleted by centrifugation at 7K for 10 minutes in a SS34 rotor and resuspended in 10 ml of 100mM NaCl, 1mM EDTA, 50mM Tris-HCl (pH 8.0) containing lysozyme (1 mg/ml) and incubated at room temperature for 20 minutes. The cells were pelleted at room temperature. The pellet was resuspended in ice-cold 100mM NaCl, 1mM EDTA, 0.1% (w/v) sodium deoxycholate, 50 mM Tris-HCl (pH 8.0) and incubated on ice with occasional mixing for 10 minutes. $MgCl_2$ and DNase I were added to a final concentration of 8 mM and 10 µg/ml, respectively. The suspension was incubated at 4°C with occasional mixing until the viscosity

disappeared. The inclusion bodies were pelleted from the suspension by centrifugation at 6000 r.p.m. for 10 minutes at 4°C. The pellet containing the inclusion bodies was washed once with 2M urea in PBS, once with PBS, suspended in 4ml PBS and stored at -70°C.

2. 2. 47. Production of specific antisera against viral gene products.

To raise specific antisera, two New Zealand white rabbits were intramuscularly immunized with 0.9mg (total protein) of each purified bacterially expressed fusion protein in 0.25ml of PBS emulsified with an equal volume of Freund's complete adjuvant. Each rabbit was boosted on days 14, 30, 60 and 90 with an emulsion of 0.5 ml (0.6 mg) of fusion protein in Freund's incomplete adjuvant. Final antisera were collected on day 100. Antisera were checked by Western blotting and immunoprecipitation.

CHAPTER 3: RESULTS

3.1. INTRODUCTION

The complete DNA sequence of equine herpesvirus 1 strain Ab4[(EHV1, Ab4p), a pathogenic UK isolate, revealed that the EHV1 genome was 150,223 base pairs in size and contained 81 open reading frames predicted to encode 76 distinct polypeptides (Telford *et al.*, 1992). Among these putative genes, there are five, 1, 2, 67, 71 and 75 which either have no or poor homologues in HSV1 and VZV (Telford *et al.*, 1992). Genes 1 and 2 located in the left terminal region of U_L, are encoded on opposing DNA strands and are predicted to encode membrane proteins of 203 and 206 amino acids respectively with very similar hydrophobicity profiles. Gene 67 in IR_S/TR_S is predicted to encode a protein of 272 amino acids. A comparable ORF, IR6, has also been identified in EHV1 Kentucky A strain (Breeden *et al.*, 1992). Genes 71 and 75 are located in the short unique region of the EHV1 genome. Gene 71 is predicted to encode a protein of M_r 80 KD. Two comparable US ORFs called EUS4 and ORF1, have been identified in Kentucky A and D strains of EHV1 respectively (Audonnet *et al.*, 1990; Colle *et al.*, 1992). They are smaller than the gene 71 ORF in EHV1 strain Ab4. Gene 75 encodes a protein of 130 amino acids which was first identified in EHV1 strain Ab1 (Elton *et al.*, 1991). At the start of this project, the products of these putative genes had not been identified and their roles in the virus life-cycle were unknown. However, the fact that these genes were not conserved in other members of the herpesvirus family and were unique to EHV1 suggested that they may play specific roles in the natural host, possibly in the determination of pathogenesis.

The aim of this project was investigation of the role of the five unique EHV1 genes in the virus life cycle *in vitro*. This was subdivided into three parts: (i) Generation of deletion mutants in each of the five genes. (ii) Characterisation of the deletion mutants with respect to the role of the gene products in the virus life cycle. (iii) Expression of the gene products to raise monospecific antisera and identification and characterisation of the gene products.

For the generation of deletion mutants in each target gene, appropriate restriction enzymes were used to remove the majority of the ORF without affecting the neighbouring genes. To aid selection of mutant virus the deleted sequence was substituted by the 4.1 kb *E . coli lacZ* gene cassette including a lacZ gene flanked by a SV40 early promotor and downstream SV40 polyadenylation sequences as a marker gene (Rixon and McLauchlan, 1990). Functional analysis of the gene products was carried out by comparing the phenotype of the deletion mutants with wild-type and revertant viruses *in vitro.* For expression of the gene products, an *E.coli* ß-galactosidase fusion protein expression system was used. The level of fusion protein expression from this system is generally high and purification of the protein relatively straight forward. Identification and characterisation of the gene products were achieved using specific antisera which were raised against the expressed fusion proteins and the relevant deletion mutant, compared to the wild-type and revertant virus.

3. 2. GENERATION OF DELETION MUTANTS IN EHV1 GENES 1, 2, 67, 71 AND 75

3. 2. 1. Cloning and subcloning of relevant EHV1 DNA fragments

To clone the genomic fragments containing each target gene, EHV1 strain Ab4 DNA was prepared from infected NBL-6 cells and digested with the appropriate restriction enzyme. The fragment predicted to contain each of the five genes was purified and separately cloned into the vector pUC19 to construct four recombinant plasmids which were designated p1/2, p67, p71 and p75. To identify these recombinants, restriction enzyme digestion and Southern blotting were employed. At least three different restriction enzymes were used to identify each recombinant plasmid and digestion patterns were analysed by electrophoresis on agarose gels. The results indicated that each of the eight recombinant plasmids had the expected restriction enzyme digestion patterns (data not shown). For further identification, the four cloned fragments were labelled with ^{32}P dCTP and hybridised to southern blotted EHV1 DNA digested with restriction enzymes. The results represented in Fig. 3 show that radio-labelled p1/2 containing an EHV1 5.1 kb StuI fragment hybridised with the EHV1 5.1kb StuI fragment which contains EHV1 genes 1 and 2 (Fig. 3, d lane 1); labelled p67 containing the

gene 67 ORF in the 5.1 kb BamHI fragment hybridised to EHV1 4.7 kb and 5.1 kb BamHI fragments which contain the TR_S and IR_S copies of gene 67, respectively (Fig. 3, c lane 1). Labelled p71 containing the IR_S copy of the gene 71 ORF in a 5.8 kb BamHI/EcoRI fragment of EHV1 DNA hybridised to a 10.5 kb BamHI fragment which includes the 5.8-kb BamHI/EcoRI fragment (Fig.3, b lane 1). Labelled p75, which contains the gene 75 ORF and a TRs copy of gene 67 in a EHV1 4.9 kb StuI fragment, hybridised with the 4.9 kb StuI fragment and the 10 kb StuI fragment containing the IRs copy of gene 67 (Fig. 3, a lane 1). To construct smaller plasmids (Sp1, Sp67, Sp71 and Sp75) which contain the cloned genes and the unique restriction enzyme (RE) sites to carry out the deletion, approprate smaller restriction fragments were further subcloned into pUC19 and were identifed by restriction enzyme digestions. The results showed that the constructed recombinants had the expected RE patterns (data not shown). The nucleotide positions of the cloned and subcloned EHV1 fragments in the recombinant plasmids are summarised in Table 2.

3. 2. 2. Construction of deletion and substitution plasmids

To construct deletion and substitution plasmids, each recombinant plasmid was separately digested with restriction enzymes which cut at unique sites to remove the majority of the coding sequences of the target genes. The flanking sequences were religated with synthetic oligonucleotides with complementary ends, which contain an unique SpeI site to allow insertion of the *lacZ* gene and an up-stream in frame stop codon to prevent synthesis of a fusion protein from the remaining N-terminal sequence and downstream *lacZ* insertion. To construct the gene 1 deletion plasmid, the majority of the gene 1 ORF (1373-1881 n.p.) was removed by AscI and HpaI digestion from Sp1/2 and the flanking regions were religated with the adaptor 1. For selection the *E. coli LacZ* gene cassette was inserted into the SpeI site in the adaptor. To construct the gene 2 deletion plasmid, the majority of the gene 2 ORF (1998-2398 n.p.) was removed by PflmI digestion and the flanking regions were religated with adaptor 2. For seletion of the mutant, the *LacZ* gene cassette was inserted into the SpeI site in adapter 2. To construct the gene 67 deletion plasmid, the majority of gene 67 ORF (124416-124929 n.p.) was removed by BsaAI and StyI digestion and the flanking regions

Figure 3. Southern blot of restriction enzyme digestion of EHV1 DNA

EHV1 DNA digestions and a kilo base pairs (kbp) molecular weight ladder were separated on a 0.8% agarose gel and transferred to a Nylon membrane, and probed with ^{32}P labelled recombinant plasmid DNA. Lane 1, EHV1 DNA digested; Lane 2, kbp ladder;

(a). StuI digested EHV1 DNA probed with p75 DNA.

(b). BamHI digested EHV1 DNA probed by p71.

(c). BamHI digested EHV1 DNA probed with p67.

(d). StuI digested EHV1 DNA probed by P1/2

EHV1 DNA bands are labelled by◁.

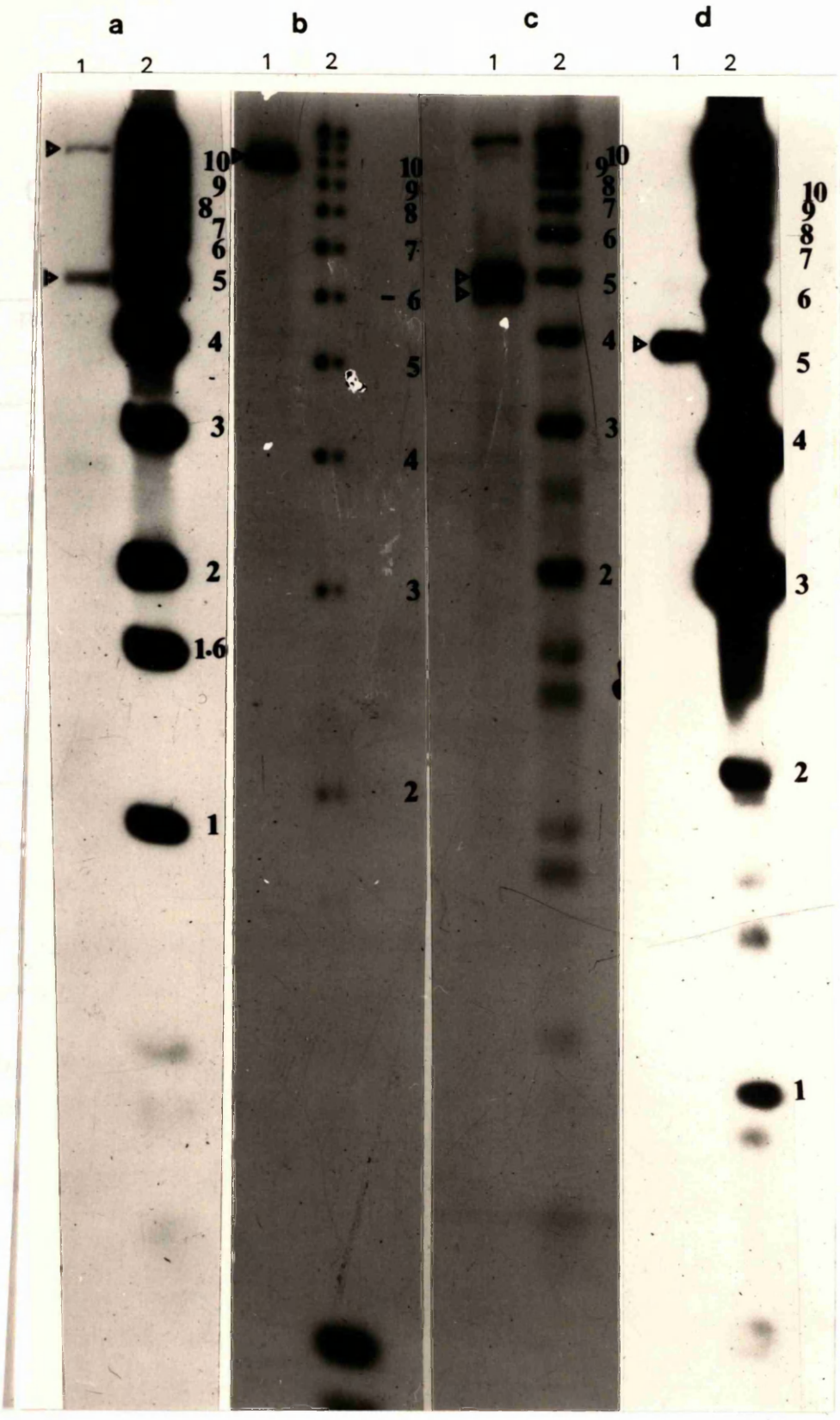

a
b
c
d
1
2
10
9
8
7
6
5
4
3
2
1.6
1

Table 2. Cloned and subcloned EHV1 fragments which contain the five target genes *

Plasmid	nucleotide position(n.p.) of the fragment	R.E. fragment + size in base pairs(bp)	target gene included	cloning site
p1/2	975-6158	StuI 5183	1 &2	HincII
Sp1/2	975-4539	StuI/PstI 3565	1 &2	StuI/PstI
p67	121400-126517	BamHI 5117	67	BamHI
Sp67	122906-125606	SalI 2700	67	SalI
p71	126517-132305	Ba/Eco 5788	71	BamHI/EcoRI
Sp71	127676-132305	PstI 4629	71	PstI
p75	134567-139452	HincII 4885	75	HincII
Sp75	134566-138168	SphI 3602	75	SphI

*The EHV1 fragments were individually cloned into the vector pUC19 polylinker site to construct these recombinant plasmids.

Ba: BamHI; Eco:EcoRI; R.E.: Restriction enzyme;

were religated with adaptor 3. For selection of the mutant, a *LacZ* gene was inserted into the SpeI site in adaptor 3. To construct a gene 71 deletion plasmid, the majority of the gene 71 ORF (129211-131022 n.p.) was removed by SgrAI and MscI digestion and the flanking regions were religated with adaptor 4. For selection of the mutant the *LacZ* gene cassette was inserted into the SpeI site in the adaptor 4. To construct the gene 75 deletion plasmid, the majority of gene 75 ORF (136115-136304 n.p.) was removed with BsmI and Bpu1102 I digestion and the flanking regions religated with adaptor 5. For selection of the deletion mutant, the *lacZ* gene cassette was inserted into the SpeI site in adaptor 5. The sequences of the synthetic oligonucleotides are summarised in Fig. 4. In this way, 10 deletion and substitution plasmids, pD1, pDL1, pD2, pDL2, pD67, pDL67, pD71, pDL71, pD75 and pDL75, were constructed. In each case the majority of the target gene ORF was removed and replaced by a *lacZ* gene [a 4.1 kb XbaI fragment of pFJ3 (Rixon and McLauchlan, 1990)] in the same orientation as the substituted gene. The construction and sequence arrangements of these deletion plasmids are presented in Figs. 5 to 9. The genome structures of the plasmids were confirmed by restriction enzyme digestion. The results showed that the constructed deletion plasmids had the expected restriction enzyme digestion patterns (data not shown). The nucleotide position and size of each deleted region in these constructed plasmids are summarised in Table 3.

3. 2. 3. Generation of deletion mutants in the target genes.

To generate deletion mutants in each target gene, monolayers of BHK21/C13 cells were cotransfected with the appropriate deletion plasmid DNA and EHV1 Ab4 DNA as described in method 2.2.6. Deletion mutant progeny virus were identified by blue staining in the presence of X-gal (see section 2.2.7). Five individual deletion mutants in genes 1, 2, 67, 71 and 75 were separately isolated and designated as ED1, ED2, ED67, ED71 and ED75, respectively. The genome structure of each deletion mutant was confirmed by Southern blotting and restriction enzyme digestion of ^{32}P-labelled viral DNA. The results are shown in Figs. 10 and 11 and diagramatically illustrated in Figs.12 and 13. In the ED1 genome, the deletion [1373-1881 nucleotide position (n.p.)] and *lacZ* substitution introduce a novel EcoRI site into

Figure 4. Sequence arrangements of synthetic oligonucleotide adaptors.
To construct deletion plasmids, pD1, pD2, pD67, pD71 and pD75, five double-stranded oligonucleotide adaptors, called adaptor 1, 2, 3, 4 and 5, were synthesised. These oligonucleotides have overhanging complementary ends as indicated and contain an in frame stop-codon (underlined) and an unique SpeI site (in bold).

Adaptor 1.

AscI 'sticky end' **HpaI 'blunt end'**

5' CGC GCC ATA AGC TAG **CAC TAG T**GA TAA CT 3' (29mer)

3' GG TAT TCG ATC **GTG ATC AC**T ATT GA 5' (25mer)

Adaptor 2.

PflmI 'sticky end' **PflmI 'sticky end'**

5' CT GGC TAG **CAC TAG T**GA TAT GTT ATACG 3' (28mer)

3' TTAG CCG ATC **GTG ATC AC**T ATA CAA TA 5' (27mer)

Adaptor 3.

BsaAI 'sticky end' **StyI 'sticky end'**

5'-CAAGTAAG TAA GTA AG**A CTA GTG** C-3' (24mer)

3'-TTC ATT CTT TC **T GAT CAC** GAACGT-5' (24mer)

Adaptor 4.

SgrAI ' sticky end' **MscI 'sticky end'**

5'- ACC TAG **GAC TAG TC**T AAC TAA CTA AC- 3' (26mer)

3'-GG ATC **CTG ATC AG**A TTG ATT GAT TGATTGCGGCC-5' (34mer)

Adaptor 5

BsmI 'sticky end' **Bpu1102 'sticky end'**

5'- C TGC TAG **CAC TAG T**AA CTA ACT AA-3' (24mer)

3'- GCG ACG ATC **GTG ATC A**TT GAT TGA TTACT-5' (29mer)

Figure 5. Schematic representation of plasmid constructed for gene 1 deletion and substitution.

Line 1, EHV1 genome consisting of U_L/U_S and inverted repeat regions (IRs/TRs) (not to scale). Line 2, expanded cloned 5.1 kb StuI fragment. Line 3, subcloned 3.5 kb StuI/PstI fragment containing genes 1, 2 and 3 ORFs. Line 4, deletion plasmid pD1 with a deletion between AscI site (1373 n.p.) and HpaI site (1881 n.p.) was linked by synthetic oligonucleotide adaptor 1. Line 5, deletion and substitution plasmid pDL1 with a lacZ gene insertion in the pD1 SpeI site. SpeI and XbaI have complementary ends, but ligation between them disrupts both XbaI and SpeI sites. P1 represents SV40 early promoter; T represents SV40 polyadenylation signal. Relevant restriction sites: As: AscI; Hp:HpaI; Ps:PstI; St:StuI.

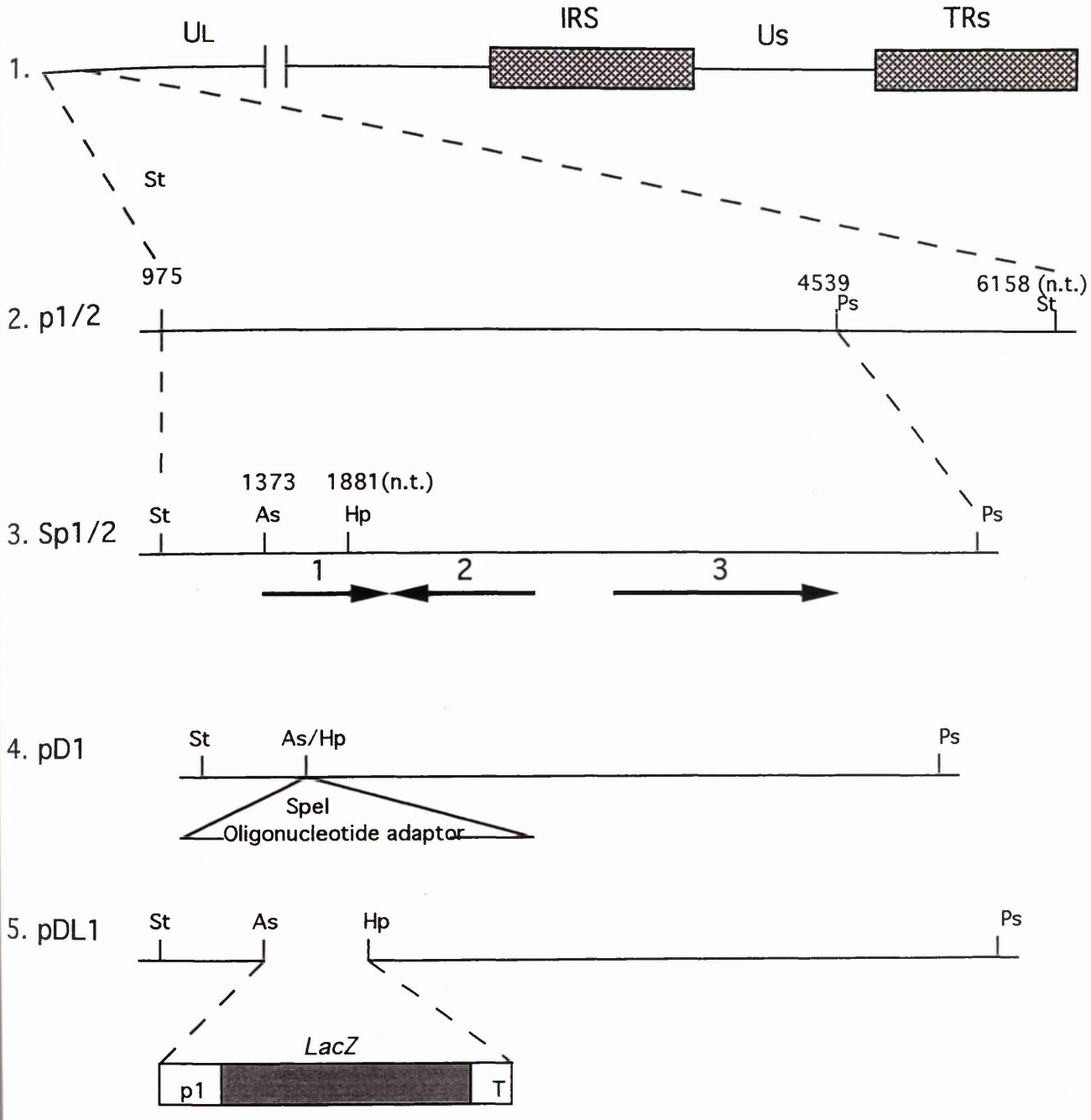

1.
UL
IRS
Us
TRs
St
975
2. p1/2
4539
Ps
6158 (n.t.)
St
1373
1881(n.t.)
3. Sp1/2
St
As
Hp
Ps
1
2
3
4. pD1
St
As/Hp
Ps
SpeI
Oligonucleotide adaptor
5. pDL1
St
As
Hp
Ps
LacZ
p1
T

Figure 6. Schematic representation of deletion plasmid constructed for gene 2.

Lines 1, 2 and 3 (see Figure 5 lines 1, 2, 3). Line 4, deletion plasmid; pD2 the sequence between two PflmI sites was removed and the flanking regions were religated by oligonucleotide adaptor 2. Line 5, deletion and substitution plasmid pDL2 with a *lacZ* insertion in the pD2 SpeI site. Relevant restriction sites: Pf: PfmlI; Ps: PstI; St: StuI. P1 and T see Figure 5 legend.

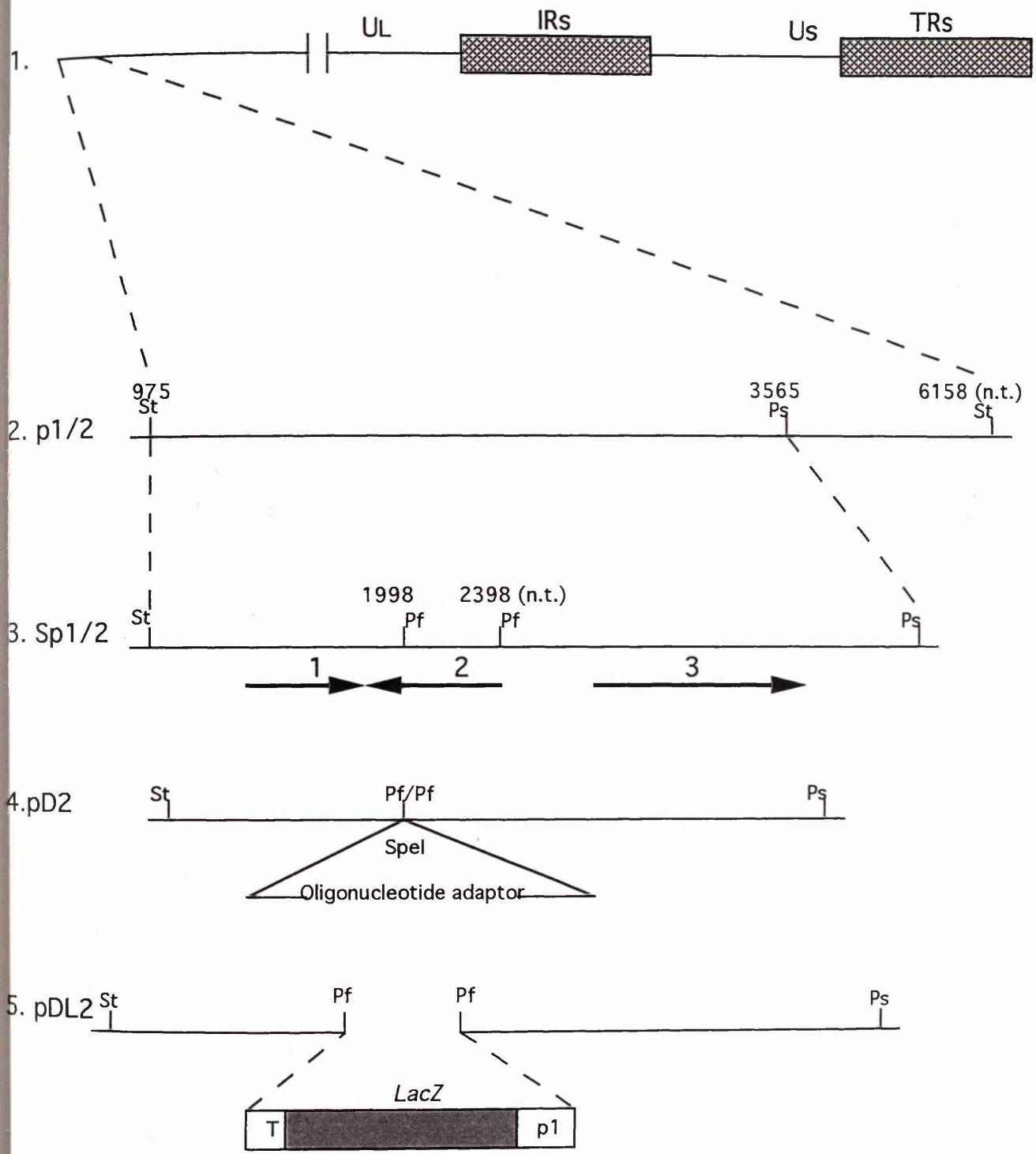

1.
UL
IRs
Us
TRs
975
St
3565
Ps
6158 (n.t.)
St
2. p1/2
1998
2398 (n.t.)
St
Pf
Pf
Ps
3. Sp1/2
1
2
3
4.pD2
St
Pf/Pf
Ps
SpeI
Oligonucleotide adaptor
5. pDL2
St
Pf
Pf
Ps
LacZ
T
p1

Figure 7. Schematic representation of plasmids constructed for deletion of gene 67.

Line 1, EHV1 genome. Line 2, cloned EHV1 5.1 kb BamHI fragment. Line 3, subcloned 2.7 kb SalI fragment containing gene 66, 67 and 68 ORFs. Line 4, deletion plasmid pD67. The sequence between BsaAI and StyI was removed and the flanking regions were religated by the oligonucleotide adaptor 3. Line 5, deletion and substitution plasmid pDL67 with a *lacZ* insertion in the SpeI site of adaptor 3. Relevant restriction enzyme sites: Ba: BamHI; Bs: BsaAI; S: StyI: Sa, SalI. P1 and T see Figure 5 legend.

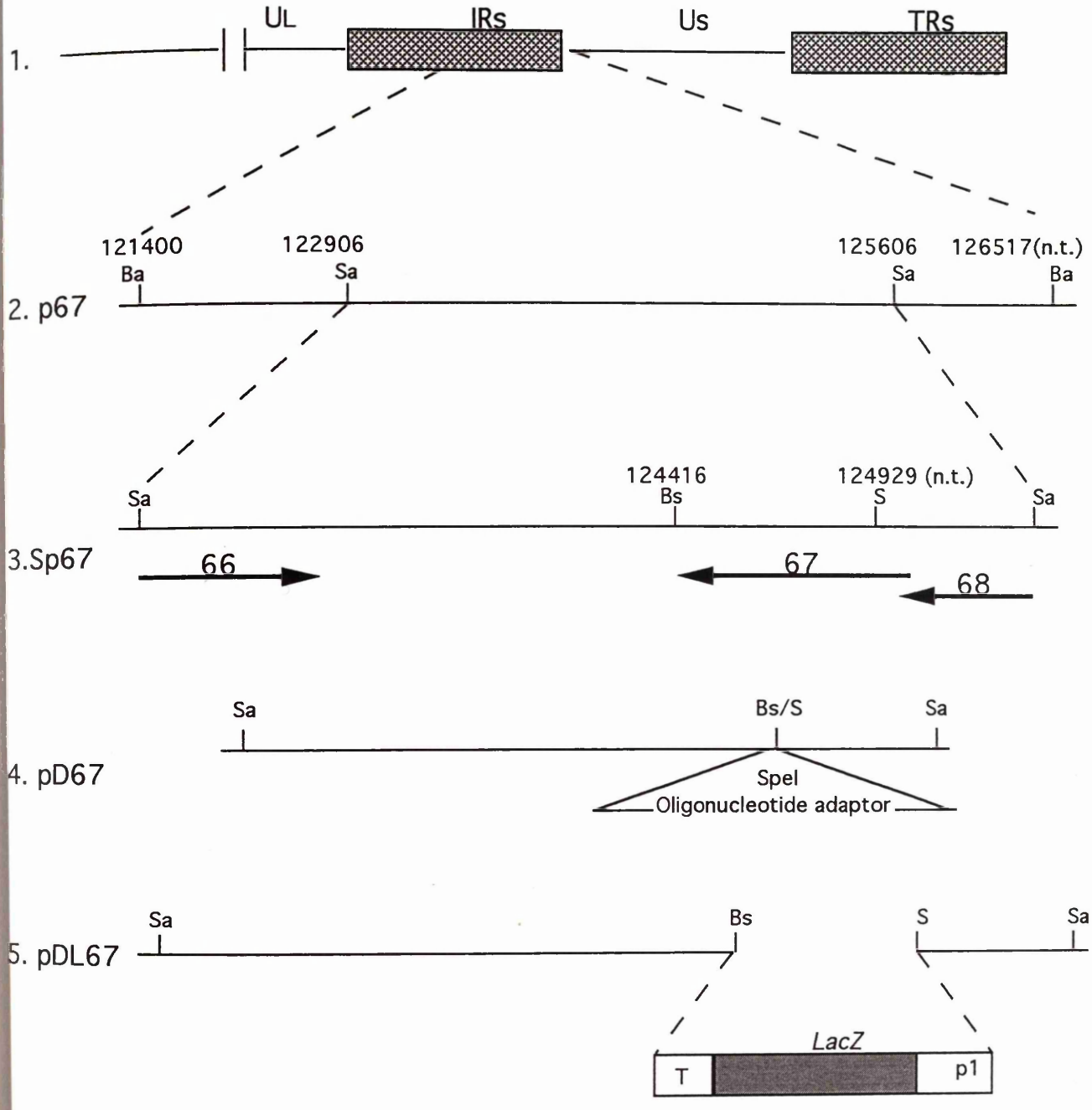

1.
UL
IRs
Us
TRs
121400
Ba
122906
Sa
125606
Sa
126517(n.t.)
Ba
2. p67
Sa
124416
Bs
124929 (n.t.)
S
Sa
3.Sp67
66
67
68
Sa
Bs/S
Sa
4. pD67
SpeI
Oligonucleotide adaptor
Sa
Bs
S
Sa
5. pDL67
LacZ
T
p1

Figure 8. Schematic representation of plasmids constructed for deletion of gene 71.

Line 1, EHV1 genome. Line 2, cloned 5.7 kb BamHI and EcoRI fragment. Line 3, subcloned 4.6 kb PstI/EcoRI fragment containing gene 70, 71 and 72 ORFs. Line 4, deletion plasmid pD71, the sequence from MscI to SgrAI was removed and the flanking regions were religated with oligonucleotide adaptor 4. Line 5, deletion and substitution plasmid pDL71 with a *lacZ* insertion in the SpeI site of adaptor 4. Pertinent restriction enzyme sites: Ba: BamHI; Ec: EcoRI; Ms :MscI; Sg: SgrAI. P1 and T see Figure 5 legend.

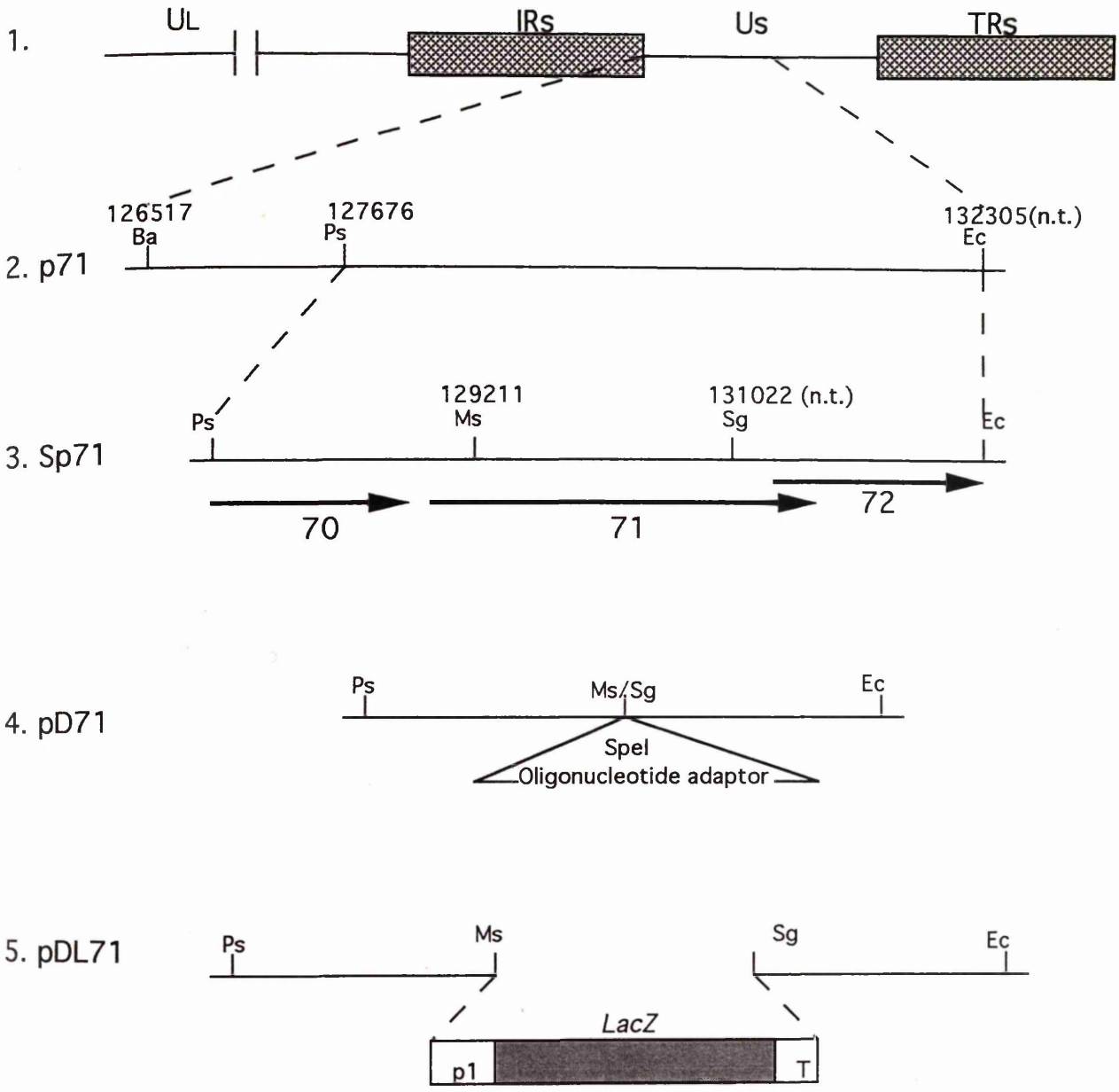

1.
UL
IRs
Us
TRs
126517
Ba
127676
Ps
132305(n.t.)
Ec
2. p71
Ps
129211
Ms
131022 (n.t.)
Sg
Ec
3. Sp71
70
71
72
4. pD71
Ps
Ms/Sg
Ec
SpeI
Oligonucleotide adaptor
5. pDL71
Ps
Ms
Sg
Ec
LacZ
p1
T

Figure 9. Schematic representation of plasmids constructed for gene 75 deletion.

Line 1, EHV1 genome. Line 2, cloned 4.8 kb StuI fragment. Line 3, subcloned 3.6 kb StuI/ SphI fragment containing gene 74, 75 and 76 ORFs. Line 4, deletion plasmid pD75, the region from BsaAI to Bpu1102I was removed and the flanking regions were religated with oligonucleotide adapter 5. Line 5, deletion and substitution plasmid pD75, a *lacZ* gene was inserted into the SpeI site in adapter 5. Relevant restriction enzyme sites: Bp: Bpu1102I; Bs: BsaAI; Sp: sphI. St: StuI. P1 and T see Figure 5 legend.

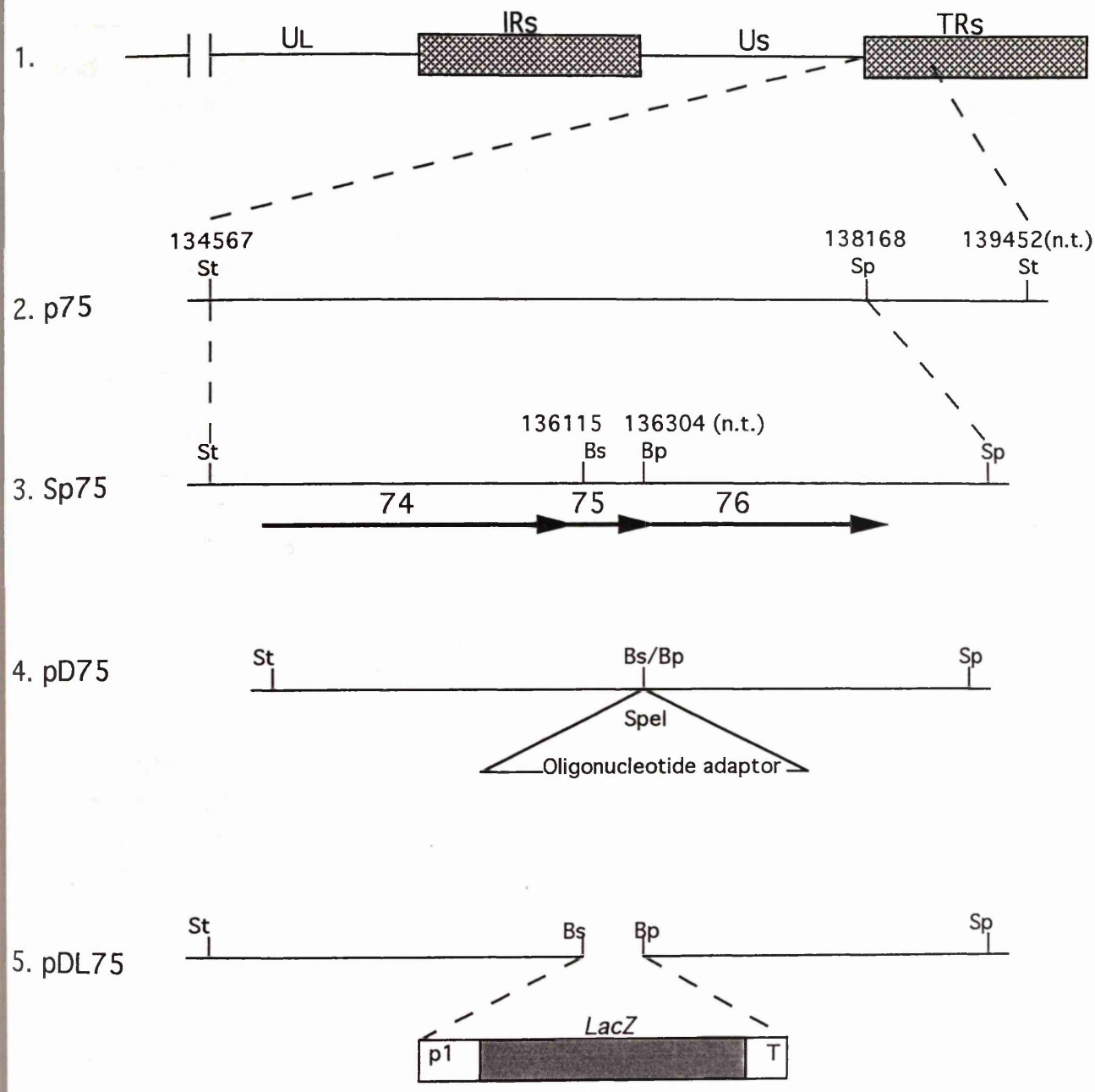

1.
UL
IRs
Us
TRs
134567
St
138168
Sp
139452(n.t.)
St
2. p75
136115
Bs
136304 (n.t.)
Bp
St
Sp
3. Sp75
74
75
76
4. pD75
St
Bs/Bp
Sp
SpeI
Oligonucleotide adaptor
5. pDL75
St
Bs
Bp
Sp
LacZ
p1
T

Table 3. Nucleotide position (n.p.) of the five target genes and the deleted regions in the constructed deletion plasmids*

plasmid	target gene			Deletion	
	name	location(n.p.) start	stop	region (n.p.)	size (bp)
pD1	1	1298	1906	1373-1881	508
pD2	2	2562	1945	1998-2398	400
pD67	67	125193	124375	124416-124929	513
pD71	71	129096	131489	129211-131022	1811
pD75	75	136054	136446	136115-136304	189

*The majority of each target gene ORF in recombinant plasmids was removed by restriction enzymes. The flanking regions were religated with an oligonucleotide adaptor and a lacZ gene was inserted into the SpeI site to construct the five deletion plasmids.

bp: base pair

the wild-type 9.1 kb EcoRI fragment which results in the replacement of the 9.1 kb fragment with two new fragments, one of 7.2 kb migrating above the 6.6 kb fragment and the other a 5.5 kb terminal fragment running between the 6.2 and 4.4 kb fragments. The 9.1 kb fragment runs just below the 9.3 kb fragment and therefore the absence of this fragment in the mutant could not be clearly seen (Fig. 10a, lane 2 and Fig. 12, a). A Southern blot of XhoI digested ED1 DNA showed that pU1/2 hybridised to the expected 5.4 kb and 2.8 kb bands but not to the wild-type 1.5 and 3.0 kb bands, indicating the mutant was not contaminated with wild-type virus (data not shown). In the mutant ED2 the deletion (1998-2398 n.p.) and *lacZ* substitution also introduce a new EcoRI site into the 9.1 kb wild-type EcoRI fragment which results in two new fragments, one of 10.7 kb migrating above the 9.5 kb wild-type fragment and another terminal fragment of 2 kb, running above the 1.8 kb wild-type fragment (Fig. 10b, lane 2 and Fig. 12, a). The 9.1 kb fragment runs just below the 9.3 kb fragment and therefore the absence of this fragment in the mutant could also not be clearly seen (Fig. 10b, lane 2). A Southern blot of XhoI digested ED2 DNA showed that pU1/2 hybridised to the two novel bands (6.18 kb and 0.49 kb), but not to the 3.07 kb wild-type virus band, confirming that mutant ED2 is not contaminated with wild-type virus (data not shown). In mutant ED67, the deletion of gene 67 (124416-124929 n.p.) and a *lacZ* insertion introduce a new SalI site into the 2.7 kb SalI fragment containing IRs and TRs copies of gene 67. This results in two new SalI fragments (1.5 kb and 4.8 kb) (Fig. 11, lanes 3 to 6 and Fig. 13, b). In mutant ED71 the deletion (129,211-131,022 n.p.) and a 4.2 kb *lacZ* substitution results in a new 6.2kb SmaI fragment replacing the 3.8kb wild-type SmaI fragment (Fig. 10, c lane 2 and Fig. 12, b). The deletion of gene 75 (136,115-136,304 n.p.) and *lacZ* substitution in the wild-type 6.3 kb SmaI fragment changes the fragment to a new 10 kb fragment running above the 9.2 kb wild-type fragment in SmaI digested ED75 DNA (Fig. 10, d lane 2 and Fig. 13, a).

In addition, to confirm that the correct region in each mutant was absent, plasmid DNA was digested by the restriction enzymes used to carry out the deletion. The deleted fragments in mutants were purified and labelled with ^{32}P and used as a probe. Southern blotting

Figure 10. Autoradiographs of restriction digests of the DNAs of mutants ED1, ED2, ED71, ED75 and EHV1 strain Ab4 labelled *in vivo* with ^{32}P.

^{32}P labelled EHV1 DNA (Lane 1) and mutant DNA (Lane 2) were separated on agarose gels and autoradiographed. ▶indicates new fragments; ■indicates missing fragments. Fragment sizes are indicated in kb to the left and the right.

(a). EcoRI digested EHV1 DNA (Lane 1) and ED1 DNA (Lane 2)

(b). EcoRI digested EHV1 DNA (Lane 1) and ED2 DNA (Lane 2)

(c). SmaI digested EHV1 DNA (Lane 1) and ED71 DNA (Lane 2)

(d). SmaI digested EHV1 DNA (Lane 1) and ED75 DNA (Lane 2)

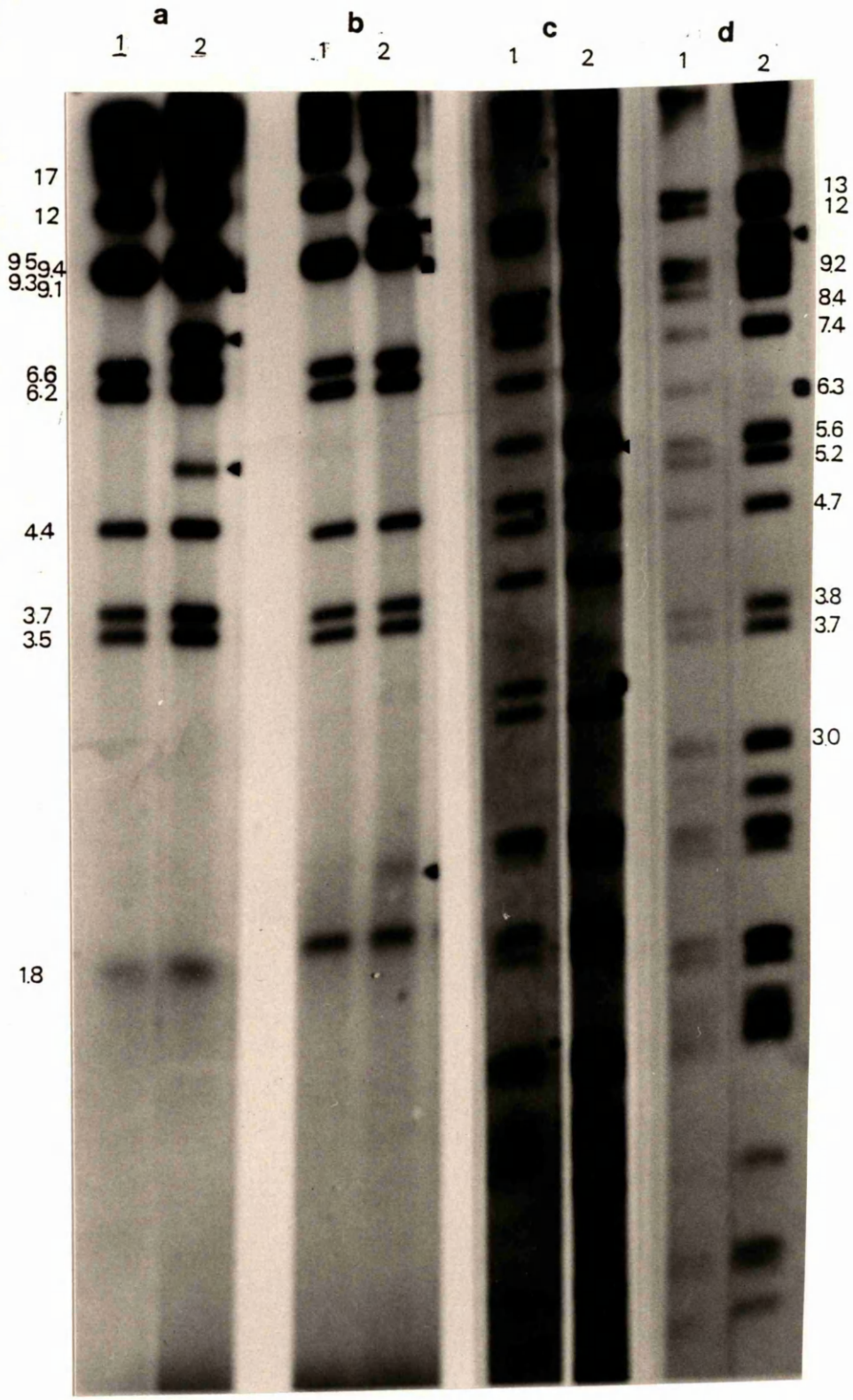

a
b
c
d
1
2
1
2
1
2
1
2
17
12
9.5
9.4
9.3
9.1
6.6
6.2
4.4
3.7
3.5
1.8
13
12
9.2
8.4
7.4
6.3
5.6
5.2
4.7
3.8
3.7
3.0

Figure 11. Autoradiographs of Southern blots of the DNA of mutant ED67 and wild-type virus.

SalI digested viral DNA was separated on a 0.8% agarose gel and transferred to a Nylon membrane, and probed with ^{32}P labelled Sp67 DNA and ^{32}P labelled 1 kb DNA marker. Lane 1, 1 kb DNA markers. Lane 2, SalI digested EHV1 DNA. Lanes 3 to 6 SalI digested ED67 DNA. Fragment sizes are indicated in kb to the right. Molecular weight markers are present on the left in KD.

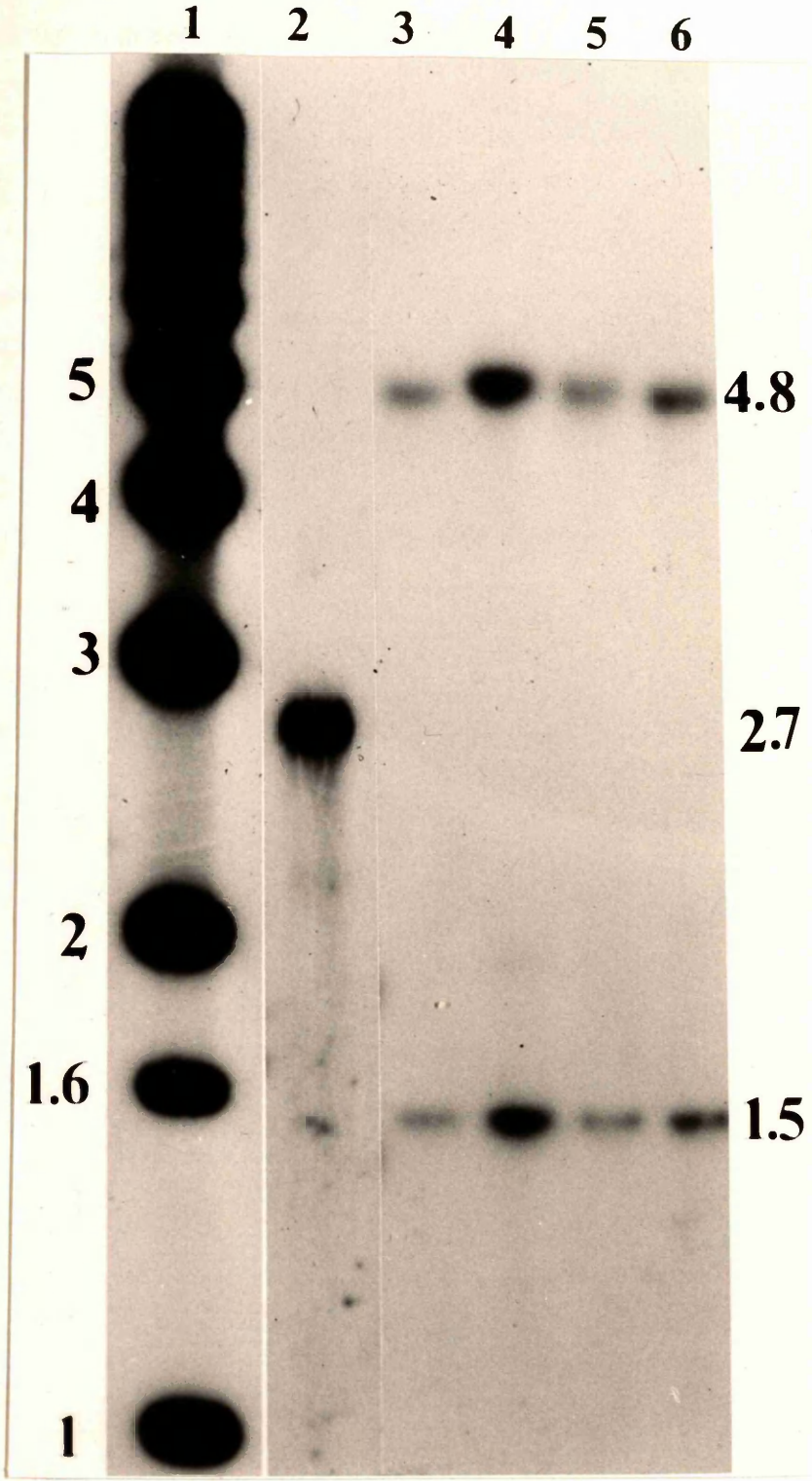
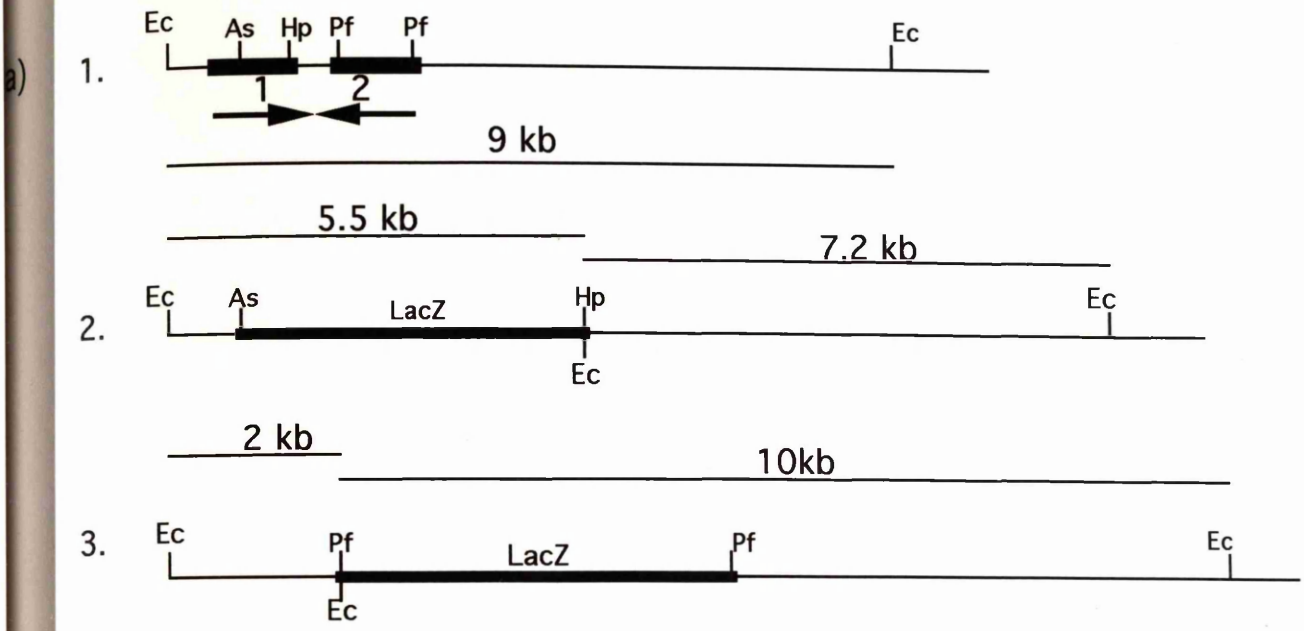

1
2
3
4
5
6
5
4
3
2
1.6
1
4.8
2.7
1.5

Figure 12. Genome structures of mutants ED1, ED2 and ED71.

Restriction enzyme sites within the region of the genome encompassing (a) genes 1 and 2, (b) gene 71 are shown. The wild-type virus genome is represented by lines 1 and 4. The genome of mutants ED1, ED2 and ED71 are represented by lines 2, 3 and 5, respectively. Relevant restriction enzyme fragments generated following digestion with EcoRI (a) and SmaI (b) are shown. Relevant restriction sites: As: AscI; Ec: EcoRI; Hp: HpaI; Ms: MscI; Pf: PflmI; Sm: SmaI; Sg: SgrAI.


a)
1.
Ec
As
Hp
Pf
Pf
Ec
1
2
9 kb
5.5 kb
7.2 kb
2.
Ec
As
LacZ
Hp
Ec
Ec
2 kb
10kb
3.
Ec
Pf
LacZ
Pf
Ec
Ec

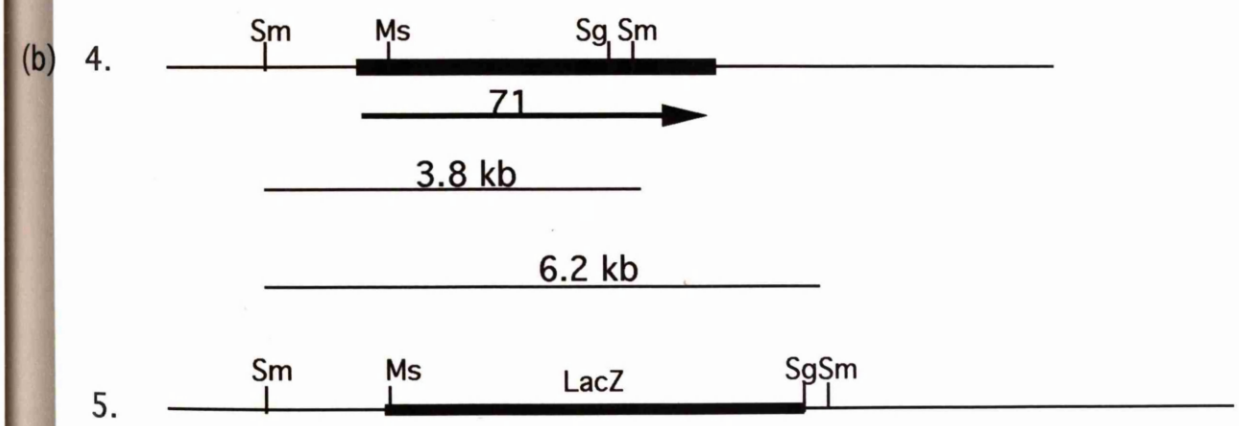
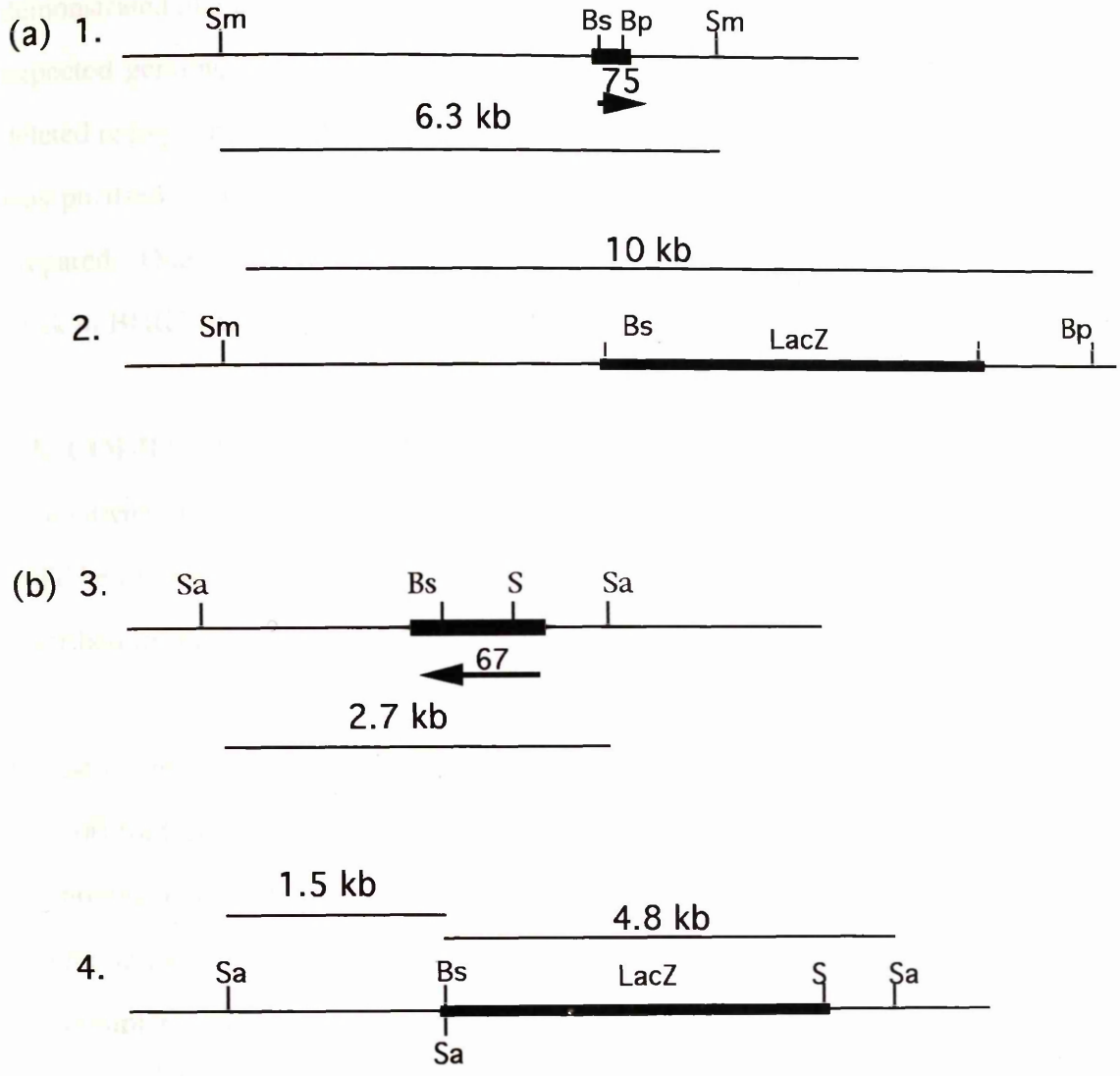

(b)
4.
Sm
Ms
Sg
Sm
71
3.8 kb
6.2 kb
5.
Sm
Ms
LacZ
SgSm

Figure 13. Genome structures of mutants ED67 and ED75.

Restriction enzyme sites within the region of the genome encompassing (a) gene 75 and (b) gene 67. The wild-type virus genome is represented by lines 1 and 3. Mutants ED75 and ED67 genomes are represented by lines 2 and 4. Relevant restriction enzyme fragments generated following digestion with SmaI (a) and SalI (b) are shown. Relevant restriction sites: Bp: Bpu1102I; Bs: BsaAI; S: StyI; Sa: SalI; Sm: SmaI.


(a) 1.
Sm
Bs Bp
Sm
75
6.3 kb
10 kb
2.
Sm
Bs
LacZ
Bp
(b) 3.
Sa
Bs
S
Sa
67
2.7 kb
1.5 kb
4.8 kb
4.
Sa
Bs
LacZ
S
Sa
Sa

experiment showed that the deleted sequences could not be detected in the relevant deletion mutant genomes by the labelled probe, but the probe hybridised to the appropriate restriction enzyme fragment in the wild-type virus genome (data not shown). These results demonstrated that the generated deletion mutants, ED1, ED2, ED67, ED71 and ED75 had the expected genome structures. The sequence arrangements and nucleotide positions of the deleted regions in the mutants are illustrated in Fig. 5 to 9 and Table 3. Each deletion mutant was purified a further four times by single plaque purification, before a virus stock was prepared. One isolate from the fourth round of purification was chosen to prepare a virus stock in BHK21/C13 cells (see section 2.2.2).

3.3. CORRECTION OF THE DELETIONS IN ED67 AND ED71

To confirm the identity of the gene 67 and 71 products and that wild-type growth properties could be restored. The wild-type genes 67 and 71 were separately rescued back as described in section 2.2.8.

To restore gene 67, BHK21/C13 monolayers were transfected with ED67 DNA and 1-, 10-, and 100-fold molar ratios of the wild-type 5.1 kb BamHI fragment in p67. To restore gene 71, monolayers of BHK21/C13 cells were transfected with ED71 DNA and 1-. 10- and 100-fold molar ratios of the wild-type virus 5.8 kb BamHI/EcoRI fragment in p71. When c.p.e. was complete, the cells were harvested and titrated on BHK21/C13 cells. The plates were incubated in the presence of X-gal (0.3mg/ml) at 37°C. After two days, single white plaques were isolated and used to infect BHK21/C13 monolayers in Linbro wells. Viral DNA was prepared and analysed by restriction enzymes as previously described (Sections 2.2.9 and 2.2.10). One isolate with a wild-type virus restriction enzyme profile was chosen from each rescue experiment (data not shown). Four rounds of plaque purification were carried out before growing up a virus stock. The revertant viruses were designated 'Re67' and 'Re71'.

3.4. GROWTH PROPERTIES OF DELETION MUTANTS AND REVERTANTS IN TISSUE CULTURE.

To investigate the phenotypes of the five deletion mutants *in vitro*, their growth properties were analysed in tissue culture under different experimental conditions and compared with those of wild-type virus and the appropriate revertant virus. Firstly, single and multi step growth curves of mutants and wild-type virus were carried out in BHK21/C13 cells at 37°C. $2x10^6$ BHK21/C13 monolayers were infected with virus either at 5 pfu/cell for single cycle growth or 0.01 pfu/cell for multi cycle growth experiments. The cells were harvested at various time points post infection. The progeny virus titres were measured on BHK21/C13 cells (Figs. 14, 15, 16 and 17). The results showed that mutants ED2, ED67 and ED75 grew to the same titres as wild-type virus in BHK21/C13 cells at 37°C with ED1 growing slightly less well than wild-type virus (Fig. 14 and 15). The deletion mutant ED71 grew more poorly than wild-type virus with the final yield of infectious virus being reduced by between 5- to 10-fold, compared with that of wild-type virus and the revertant, Re71 (Fig. 16 and 17). An interesting observation was that ED71 gave higher titres of infectious virus over the first 24 h p.i. at low m.o.i. (Fig. 17). This observation is analysed in greater detail later (Section 4.7.). ED67, revertant Re67 and wild-type virus grew identically (Fig. 14, 15 and data not shown).

Secondly, the mutants were tested for temperature sensitivity at different incubation temperatures (31°C, 37°C and 38.5°C). The results presented in Table 4 showed that mutants ED1, ED2, ED67, ED 71 and ED75 did not display any temperature sensitivity, compared with wild-type virus at the tested temperatures.

Finally, the mutants were tested in different cell lines (NBL-6, CV1, HFL1 and RK13) for host range defects. The results presented in Table 5 showed that mutants ED1, ED2, ED67 and ED75 have similar growth properties to wild-type virus in all tested cell lines whereas ED 71 displays the same level of growth defect in all cell lines as seen with BHK21/C13 CELLS.

Figure 14. Low multiplicity of infection growth experiment of ED1, ED2, ED67, ED75 and wild-type virus.

Low multiplicity of infection growth experiments with EHV1 in BHK21/C13 cells.

Monolayers of BHK21/C13 cells were separately infected with ED1, ED2, ED67, ED75 and wild-type virus at 0.01 pfu/cell. After 60 min absorption, the cells were washed three times with PBS , overlaid with Eagle's medium containing 5% calf serum, and incubated at 37°C. The progeny virus was harvested at 0, 4, 8, 12, 24, 36, 48 and 72h p. i. and titrated on BHK21/C13 cell at 37°C. The results are presented as pfu/10^6 cells.

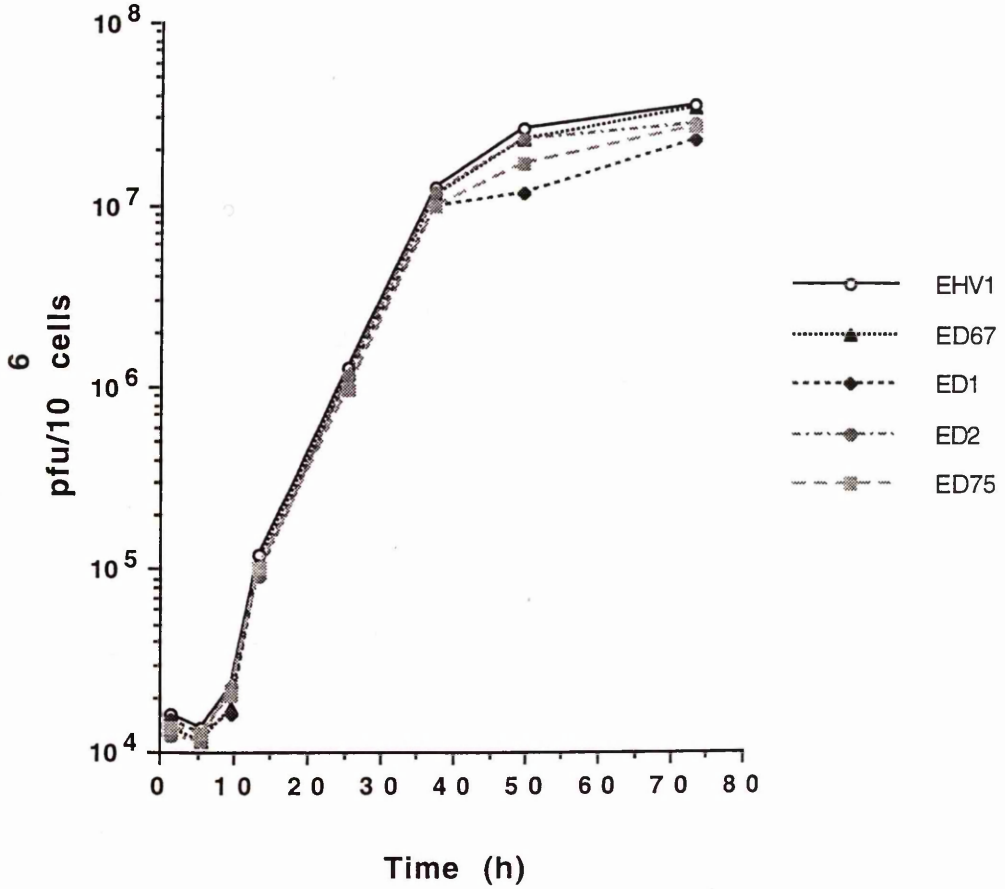

10 8
10 7
10 6
10 5
10 4
6
pfu/10 cells
0
10
20
30
40
50
60
70
80
Time (h)
EHV1
ED67
ED1
ED2
ED75

Figure 15. High multiplicity of infection growth experiment of ED1, ED2, ED67, ED75 and wild-type virus

High multiplicity of infection growth experiment of EHV1 in BHK21/C13 cells. Monolayers of BHK21/C13 cells were separately infected with ED1, ED2, ED67, ED75 and wild-type virus at 5 pfu/cell. After 60 min absorption, the cells were washed three times with PBS , overlaid with Eagle's medium containing 5% calf serum, and incubated at 37°C. Cultures were harvested at 0, 2, 8, 12, 18, 24 and 30 h p.i. The progeny virus was titrated on BHK21/C13 cells at 37°C. The results are presented as pfu/10^6 cells.

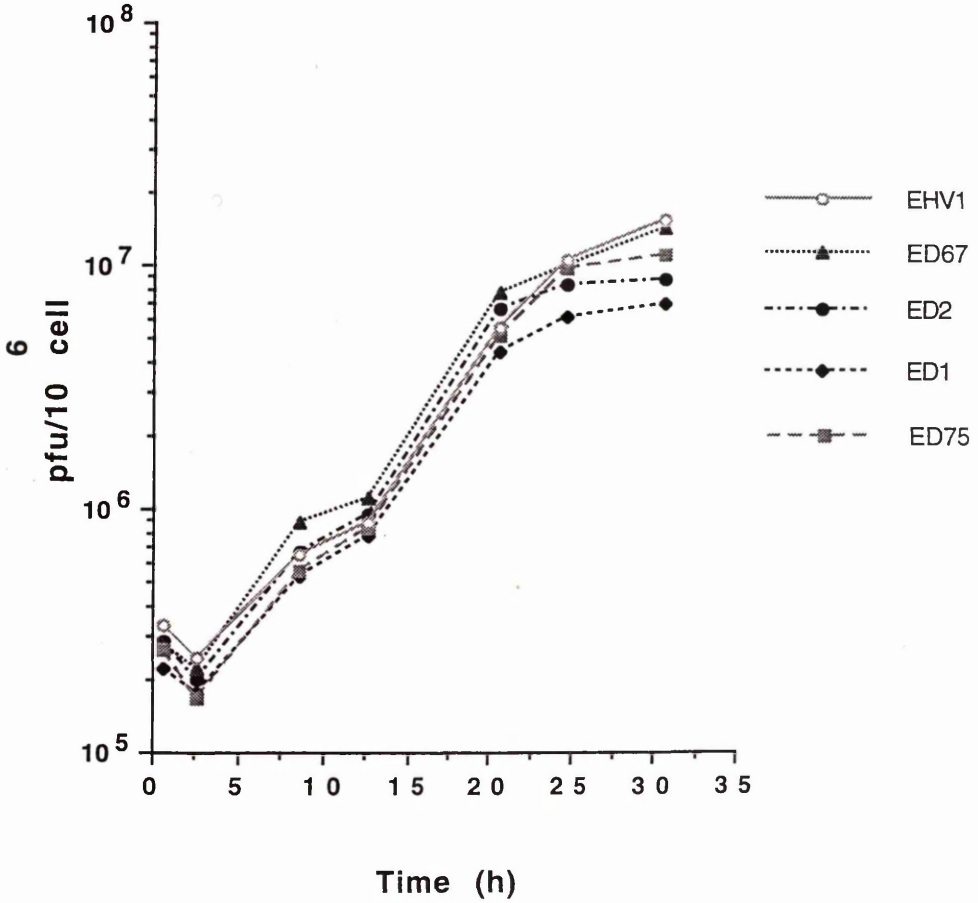

10 8
10 7
10 6
10 5
6
pfu/10 cell
0
5
10
15
20
25
30
35
Time (h)
EHV1
ED67
ED2
ED1
ED75

Figure 16. Low multiplicity of infection growth experiment of ED71, Re71 and wild-type virus.

Low multiplicity of infection growth experiment of EHV1 in BHK21/C13 cells. Monolayers of BHK21/C13 cells were separately infected with ED71, Re71 and wild-type virus at 0.01 pfu/cell. After 60 min adsorption, the cells were washed three times with PBS, overlaid with Eagle's medium containing 5% calf serum, and incubated at 37°C. Cultures were harvested at 0, 4, 8, 12, 24, 48, 72, 96 and 120 (h) p.i. The progeny virus was titrated on BHK21/C13 cells at 37°C and the titres presented as pfu/10^6 cells.

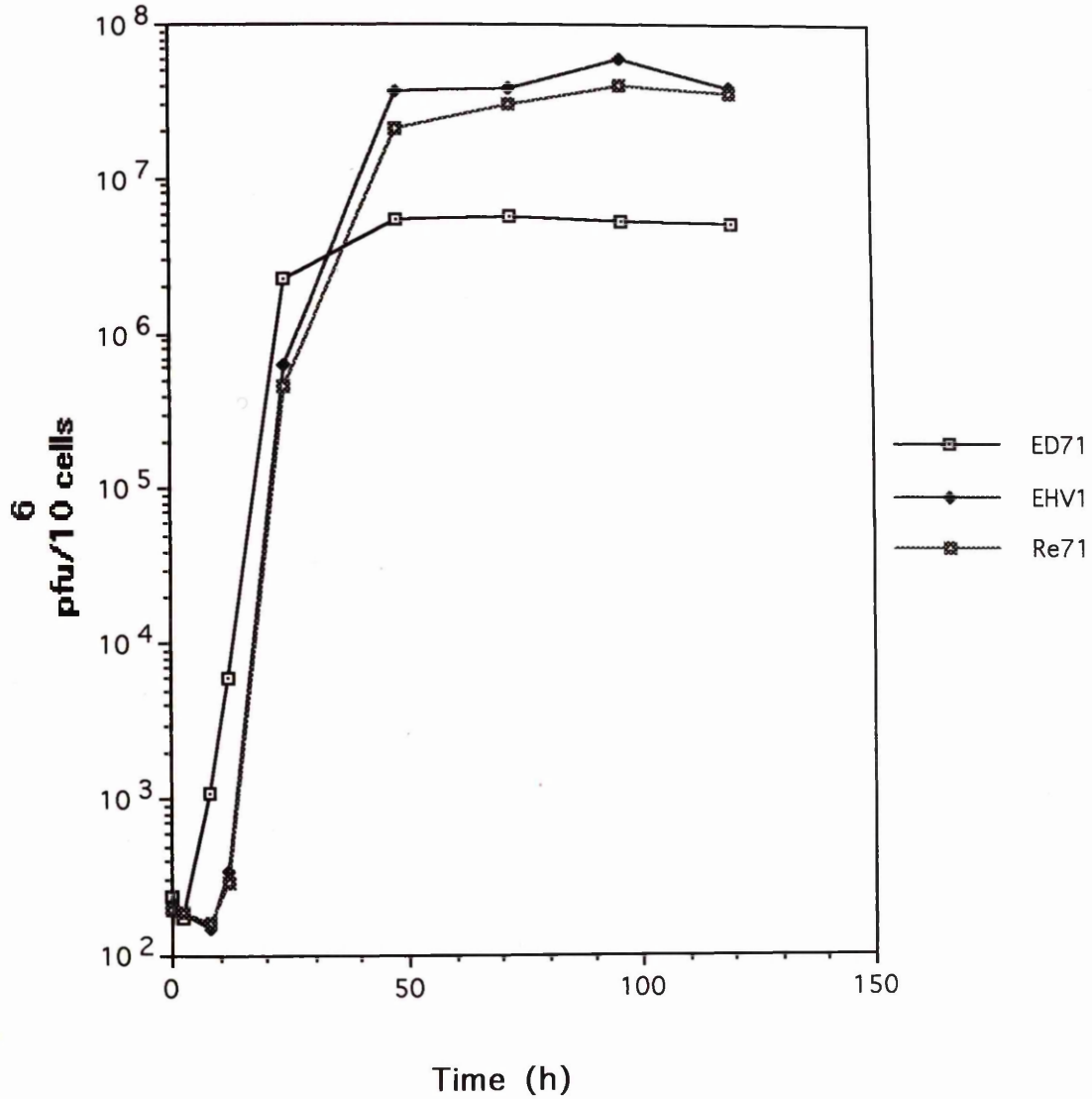

10 8
10 7
10 6
10 5
10 4
10 3
10 2
6
pfu/10 cells
0
50
100
150
Time (h)
ED71
EHV1
Re71

Figure 17. High multiplicity of infection growth experiment of ED71, Re71 and wild-type virus.

High multiplicity of infection growth experiment of EHV1 in BHK21/C13 cells. Monolayers of BHK21/C13 cells were separately infected with ED71, Re71 and wild-type virus at 5 pfu/cell. After 60 min absorption, the cells were washed three times with PBS, overlaid with Eagle's medium containing 5% calf serum, and incubated at 37°C. Cultures were harvested at 0, 4, 6, 8, 10, 12, 14, 16, 20, 24 and 30 h p.i. The progeny virus was titrated on BHK21/C13 cells at 37°C and the titres are presented as pfu/10^6 cells.

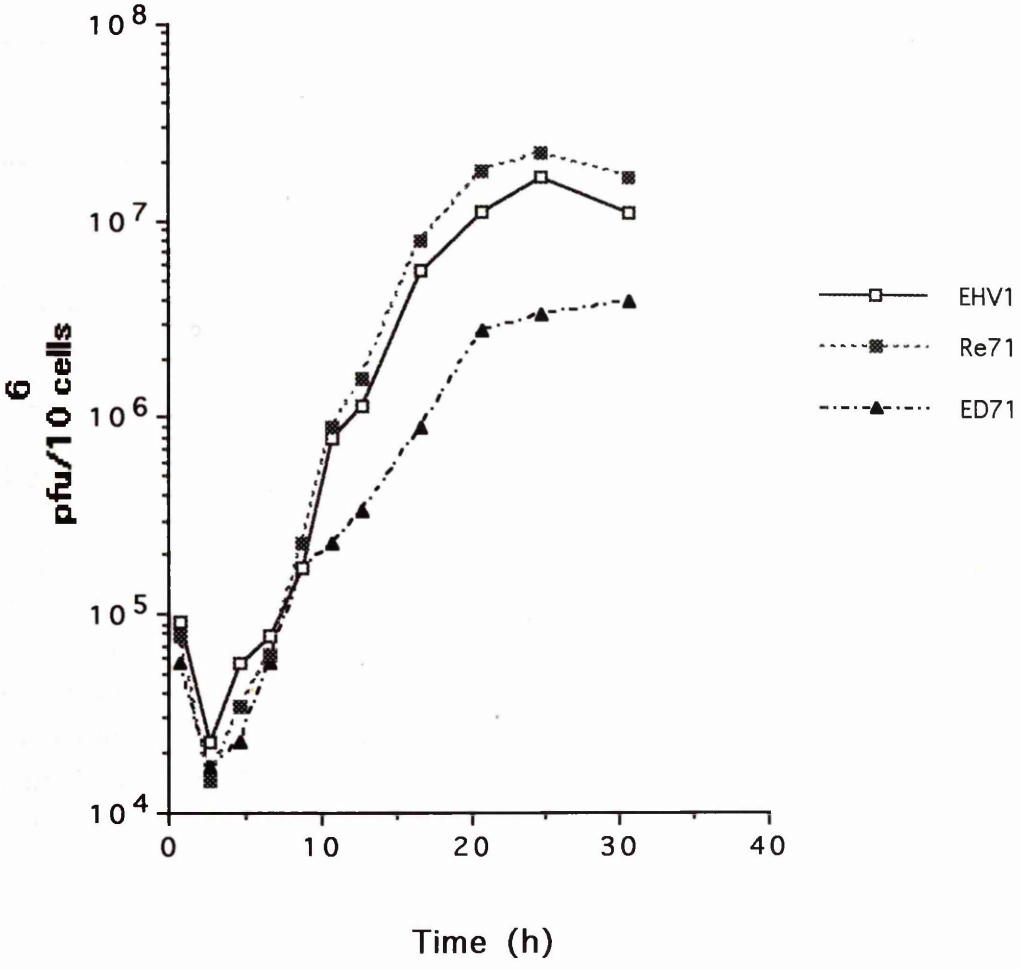

6
pfu/10 cells
10 8
10 7
10 6
10 5
10 4
0
10
20
30
40
Time (h)
EHV1
Re71
ED71

Table 4. Growth properties of the deletion mutants at three different temperatures*

Incubation temperature	Titre (pfu/ml)					
	EHV1	ED1	ED2	ED67	ED71	DE75
31°C	2.5×10^{6}	1.4×10^{6}	1×10^{6}	1.2×10^{6}	6×10^{5}	1×10^{6}
38.5°C	1.6×10^{6}	1×10^{6}	8×10^{5}	9.5×10^{5}	1.5×10^{5}	9×10^{5}
37°C	3×10^{6}	2.5×10^{6}	1×10^{6}	2×10^{6}	4.4×10^{5}	1.5×10^{6}
Ratio of titres	**pfu/pfu**					
31°C/38.5°C	1.56	1.4	1.25	1.26	4	1.1
31°C/37°C	0.8	0.56	1	0.6	1.3	0.66

*To test whether the mutation affected the temperature stability of virus, monolayers of BHK21/C13 cells in 50 mm diameter dishes were infected with virus at 5pfu/cell and incubated for 24 h at the indicated temperatures. The progeny virus was harvested and titrated on the BHK21/C13 cells at 37°C.

Table 5. Growth properties of the five mutants in various cell lines*

Cell line	Titre (pfu/ml)					
	EHV1	ED1	ED2	ED67	ED71	ED75
CV-1	$1x10^6$	$1x10^6$	$2x10^6$	$1.5x10^6$	$2.2x10^5$	$6x10^5$
HFL1	$2.5x10^6$	$2x10^6$	$2x10^6$	$3x10^6$	$4x10^5$	$2x10^6$
RK13	$1x10^6$	$1x10^6$	$9x10^5$	$1.1x10^6$	$2x10^5$	$1x10^6$
NBL-6	$4.8x10^6$	$4x10^6$	$3.6x10^6$	$5x10^6$	$6.6x10^5$	$2 x10^6$

*Monolayers of indicated cell lines in 50 mm diameter dishes were infected with virus at a m.o.i. of 5 pfu/cell and incubated for 24 h at 37°C. The progeny viruses were titrated on BHK21/C13 cells at 37°C.

Overall these results demonstrated that when the five genes were independently deleted, they are non-essential for virus growth *in vitro* at least in the tested cell lines. Deletion of the genes 1, 2, 67 and 75 does not result in any defect in host range and temperature stability. ED71 displays defective growth in all tested cell lines, compared with wild-type virus. Revertants Re67 and Re71 are indistinguishable from wild-type virus. The Re71 result indicates that the growth defect of ED71 results from deletion of gene 71. Further characterisation of ED71 is described in Section 3.8.

It should be noted that the five deletion mutants were also tested for infectivity, immunogenesis and pathogenesis in a well established murine model. This study was carried out by Tim Fitzmaurice in Cambridge University Veterinary School (see Section 4.7).

3. 5. EXPRESSION OF THE FIVE GENE PRODUCTS AS FUSION PROTEINS IN *E. COLI* AND PRODUCTION OF SPECIFIC ANTIBODIES AGAINST THE GENE PRODUCTS.

3. 5. 1. Construction of expression plasmids.

To construct fusion protein expression plasmids, the ORFs of the five genes were independently cloned into a set of pUR vectors (Rüther and Müller-Hill, 1983) to fuse in-frame with the 3' end of the ß-galactosidase gene. To construct a gene 1 protein expression plasmid, an EHV1 fragment (1329-1927 n.p.), which encodes amino acids 4-202 of the putative gene 1 protein, was amplified by PCR from Sp1/2 with two primers containing BamHI and HindIII sites, and inserted, into pUR278 BamHI and HindIII sites to fuse in-frame with the ß-galactosidase gene (Fig 18). To construct a gene 2 protein expression plasmid, an EHV1 HindII/BamHI fragment (1184-2555 n.p.) in Sp1/2, which encodes amino acids 2-205 of the putative gene 2 protein, was cloned into pUR278 BamHI and HindIII sites in frame to fuse with ß-galactosidase gene from Sp1/2 (Fig. 19). To construct the gene 67 expression plasmid, an EHV1 fragment (124411-125160 n.p.) in Sp67, which encodes amino acids 12-260 of the putative gene 67 protein, was inserted into pUR288 BamHI and

HindIII sites in frame to fuse with the ß-galactosidase gene (Fig. 20). To construct gene a 71 protein expression plasmid an EHV1 HindIII fragment (130487-131487 n.p.), which encodes 464-797 amino acids of the gene 71 protein, was inserted into pUR278 in frame to fuse with the ß-galactosidase gene (Fig. 21). To construct a gene 75 expression plasmid, an EHV1 fragment (136095-136910 n.p.), which encodes amino acids 14-130 of the putative gene 75 protein, was inserted into pUR288 BamHI and HindIII sites in frame fusing with the ß-galactosidase gene (Fig. 22). In this way five recombinant expression plasmids were constructed and designated as pU1HE, pU2HE, pU67HE, pU71HE and pU75HE. The expressed encoding sequences of the target genes in the recombinant plasmids are summarised in Table 6. The DNA profiles of the recombinant plasmids were analysed by a series of restriction enzyme digestions. Results showed that all recombinant plasmids had the expected restriction enzyme digestion patterns (data not shown). Sequence analysis of the junction between the ß-galactosidase gene and the insertions in each constructed plasmid with a primer (5'-GAATTCCAGCTGAGCGCCGGT-3') derived from the upstream sequence of the pUR cloning sites (Rüther and Müller-Hill, 1983) confirmed that the inserted amino acid encoding sequences were fused in-frame with the 3' end of the ß-galactosidase gene in the pUR vectors (data not shown).

3. 5. 2. Expression of the protein products of the genes as ß-galactosidase fusion proteins in *E. coli.*

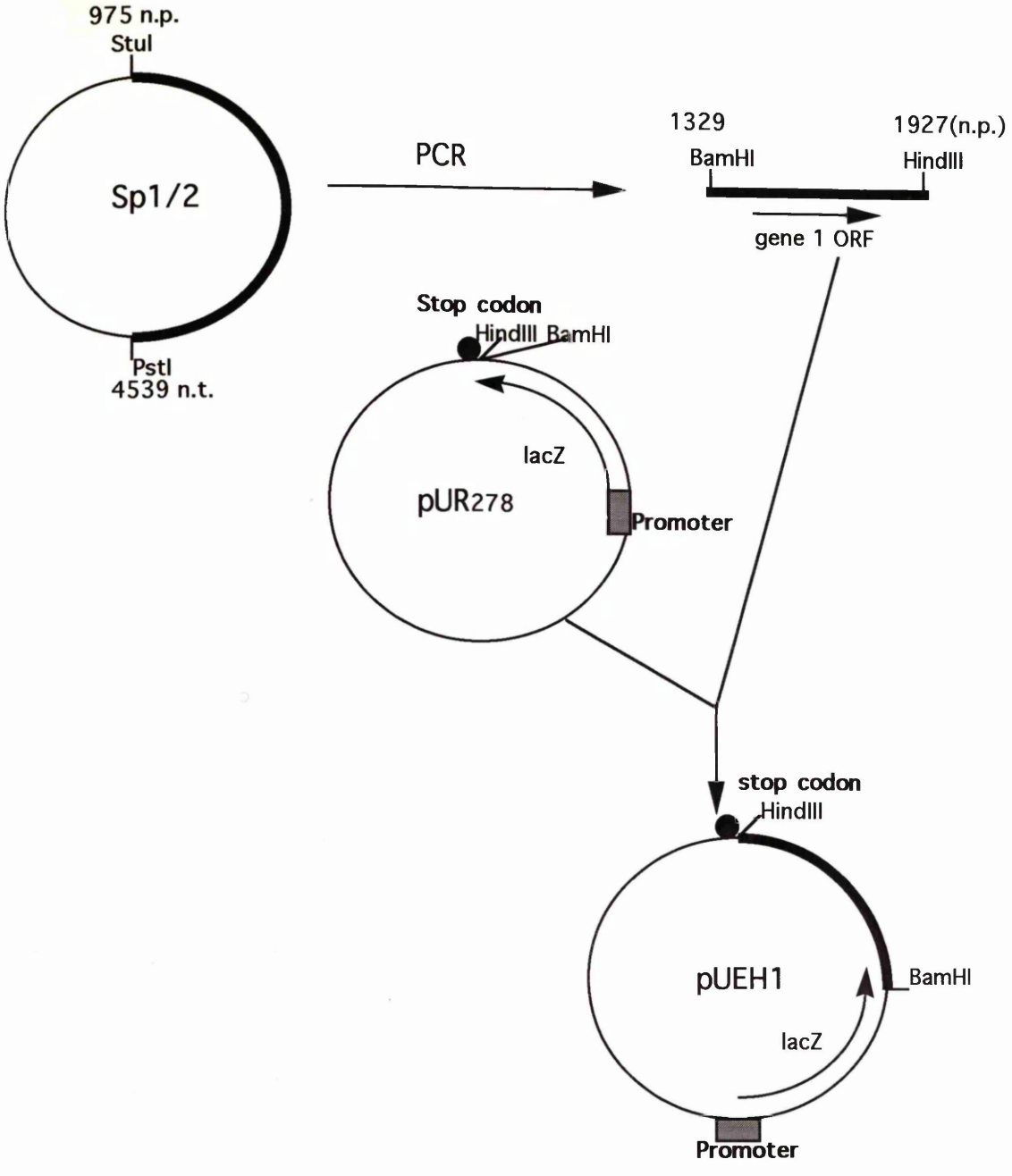
The constructed expression plasmids, pU1HE, pU2HE, pU67HE, pU71HE and pU75HE, were separately transformed into *E. coli* strain XL1 to express the fusion proteins. After induction by 1mM of IPTG, the transformed cells were lysed and the polypeptides were analysed by 6% SDS-PAGE. The results represented in Fig 23 showed that the wild-type ß-galactosidase was expressed as a 116 KD thick band (Fig. 23, lane 5) and the five fusion proteins were expressed from the transformed cells as higher M_r bands (Fig. 23). These expressed fusion proteins are designated as FP1 (lane 4), FP2 (lane 7), FP67 (lane 2), FP71 (lane 3), and FP75 (lane 1), respectively. FP1 and FP2 expressed in pUEH1 and pU2EH transformed cells consisted of the N-terminus of ß-galactosidase fused to amino acids 4-

Figure 18. Construction of expression plasmid pUEH1

An EHV1 fragment (1329-1927 n.p.) which encodes amino acid 4-202 of the gene 1 protein was amplified by PCR with primers:

(1) 5'-ATCATAGGATCCTCCTCTGTCTCCATCTCCAT-3' containing a BamHI site (underlined);

(2) 5'-TGCGATTAAGCTTTGCGGTACTACAGT-3' containing a HindIII site (underlined). from Sp1/2 with Vent polymerase under the conditions: 1 cycle of 95°C for 5 min; 30 cycles of 95°C for 30 seconds, 58°C for 40 seconds and 72°C for 1 min. The predicted 598 bp fragment was digested by BamHI and HindIII and cloned into pUR278 BamHI/HindIII sites to construct pUEH1. In pUEH1, the gene 1 coding sequence is fused in frame with the 3' end of the ß-galactosidase gene. The junction sequence between *lacZ* and the gene 1 protein encoding sequence in pUEH1 is shown. The arrow indicates the junction. The insertion site (BamHI site) is shown in bold. Thicker lines show cloned EHV1 fragments. Arrows show the orientation of transcription of genes.



The sequence of the junction between lacZ and gene 1 insertion in pUEH 1

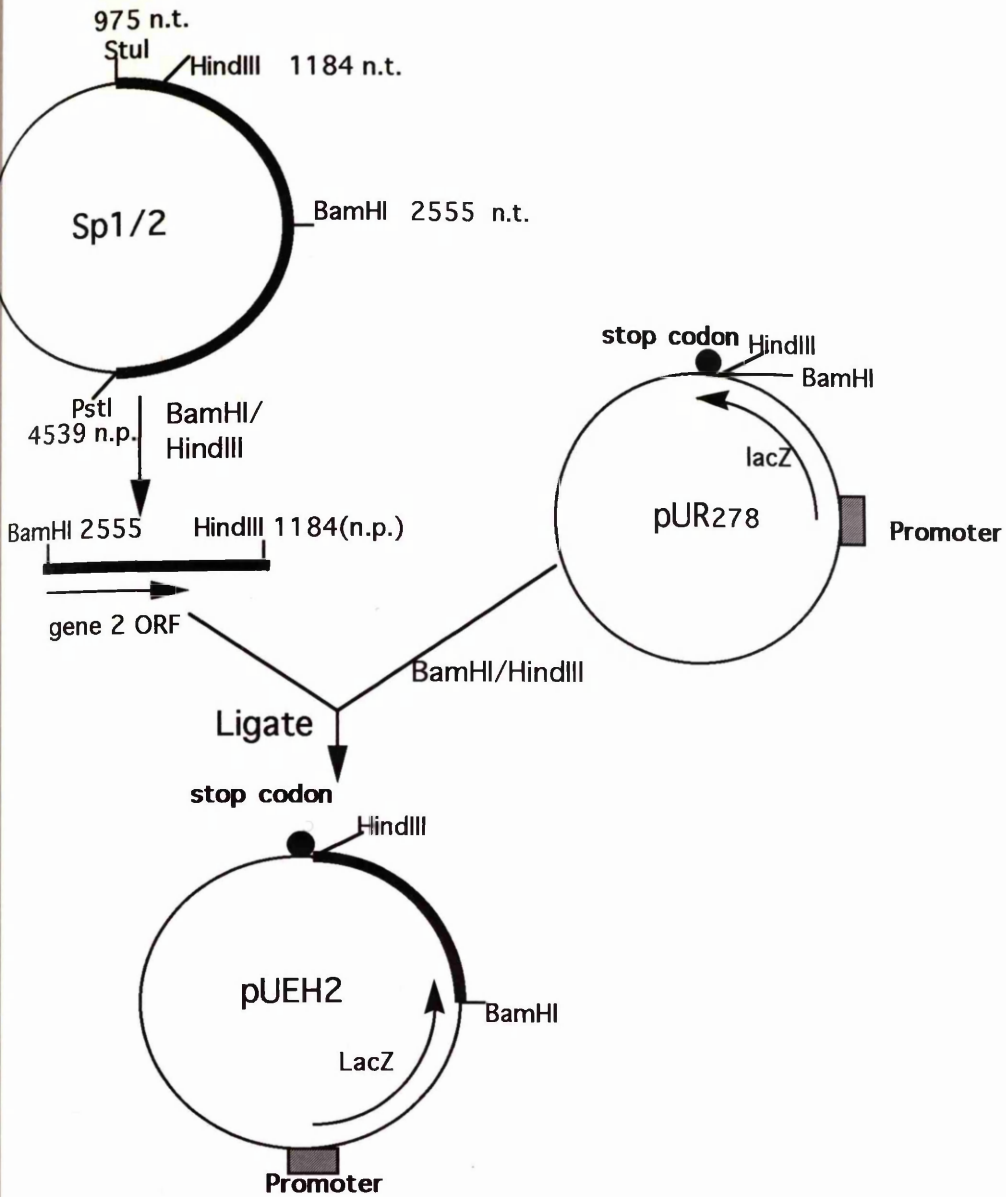
PUR278 BamHI gene 1 encoding sequence

-----CAA AAA GGG GAT CCT CCT CTG TCT CCA TCT CCA T-----

-----GTT TTT CCC CTA GGA GGA GACAGA GGT AGA GGT A-----

Figure 19. Construction of expression plasmid pUEH2.

An EHV1 BamHI/HindIII fragment (1184-2555 n.p.) encoding amino acids 2-205 of the gene 2 protein was cloned into pUR278 BamHI/HindIII sites from the plasmid Sp1/2 which contains EHV1 StuI fragment (975-4539 n.p.) to construct plasmid pUEH2. In pUEH2 the gene 2 encoding sequence is fused in frame with the 3' end of the ß-galactosidase gene. The junction sequence between *lacZ* and the gene 2 encoding sequence in pUEH2 is shown. A arrow indicates the junction and the insertion site (BamHI site) is shown in bold. Arrows show the orientation of transcription of the ß-galactosidase gene.



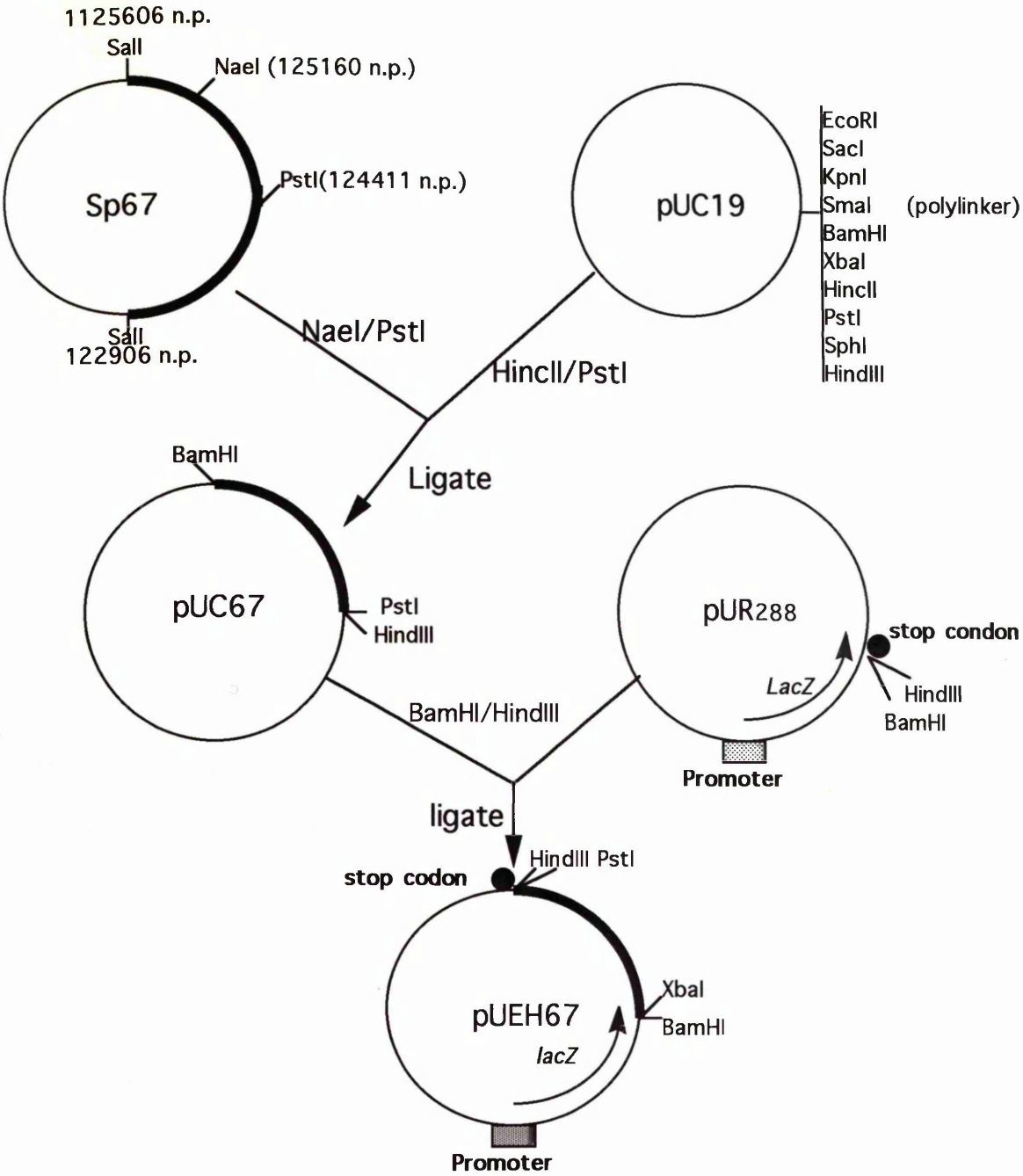
The sequence of the junction between lacZ and insertion in pUEH2

pUR278 BamHI gene 2 encoding sequence

-----CAA AAA GGG **GAT CCA** GCG TGG AGG AGG CGC CAT-----
-----GTT TTT CCC **CTA GGT** CGC ACC TCC TCC GCG GTA-----

Figure 20. Construction of expression plasmid pUEH67.

A NaeI/PstI fragment (125160-124411 n.p.) (thicker line) from the plasmid Sp67 which contains the EHV1 SalI fragment (122906-125606 n.p.) (thicker line) was cloned into pUC19 HincII/PstI sites to construct plasmid pUC67. The BamHI/ HindIII fragment in pUC67 containing the NaeI/PstI fragment was cloned into pUR288 BamHI/HindIII sites to construct pUEH67. In pUEH67 the gene 67 encoding sequence is fused in frame with the 3' end of the ß-galactosidase gene to generate pUEH67. The junction sequence between *lacZ* and the gene 67 protein encoding sequence is shown. The arrow indicates the junction and the insertion site (BamHI) is shown in bold. The arrows show the orientation of transcription of genes.



Junction between *lacZ* and gene 67 fragment in pUEH67

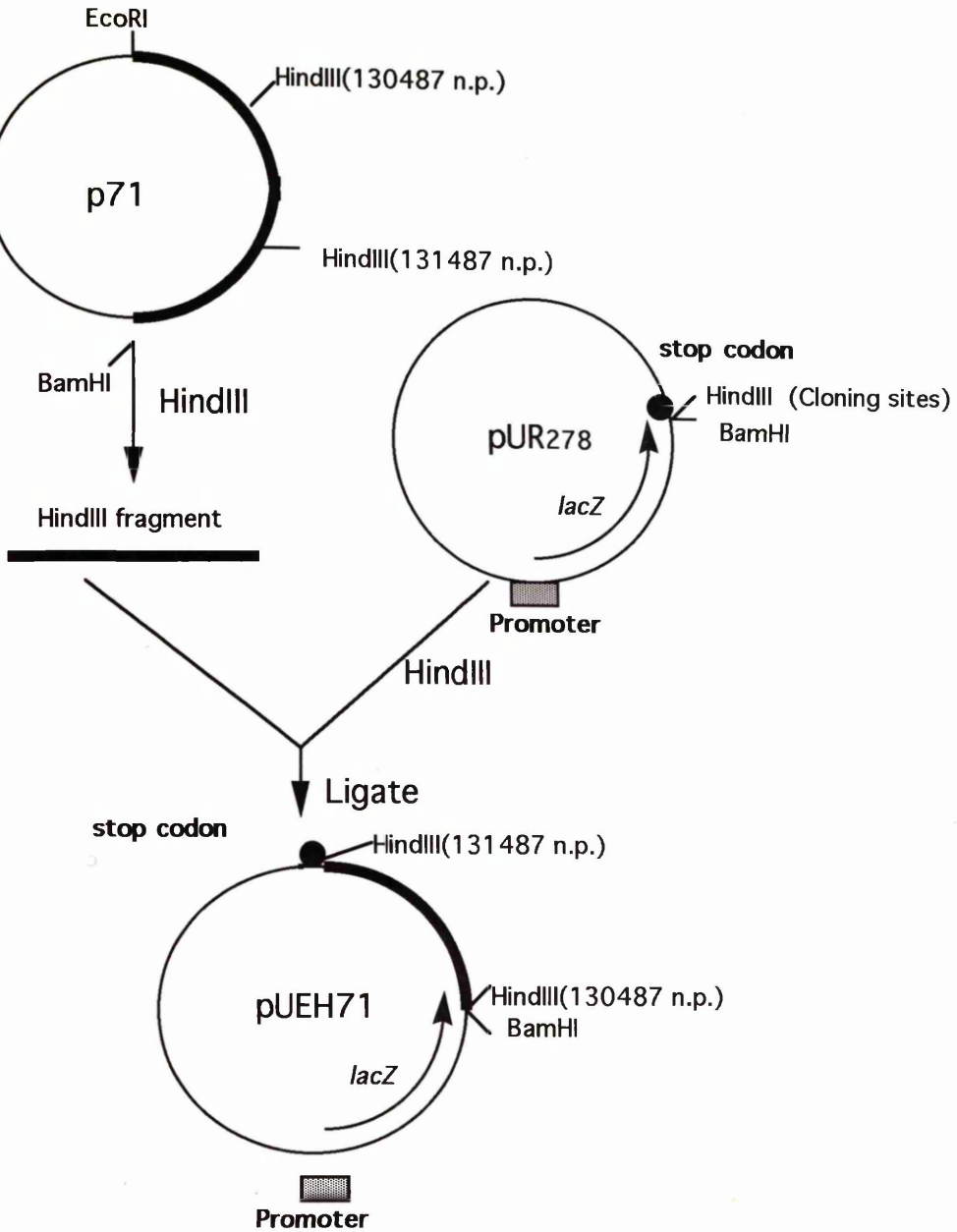
LacZ BamHI gene 67 encoding sequence

----GG **GGA TCC** TCT AGA GTC GGC ACC GAG----

----CC **CCT AGG** AGA TCT CAG CCG TGG CTC----

Figure 21. Construction of expression plasmid pUEH71.

The HindIII fragment (130487-131487 n.p.) containing the coding sequence of 464-797 amino acids of the gene 71 protein was cloned into the vector pUR278 HindIII site from the plasmid p71, which contains an EHV1 EcoRI/BamHI fragment (thicker line), to construct pUEH71. In pUEH the gene 71 coding sequence was fused in frame with the 3' end of the ß-galactosidase gene to generate pUEH71. The sequence of the junction between *lacZ* and the gene 71 protein encoding sequence is shown. The arrow indicates the junction and the insertion site (BamHI) is shown in bold. The arrows show the orientation of the transcription of the genes.



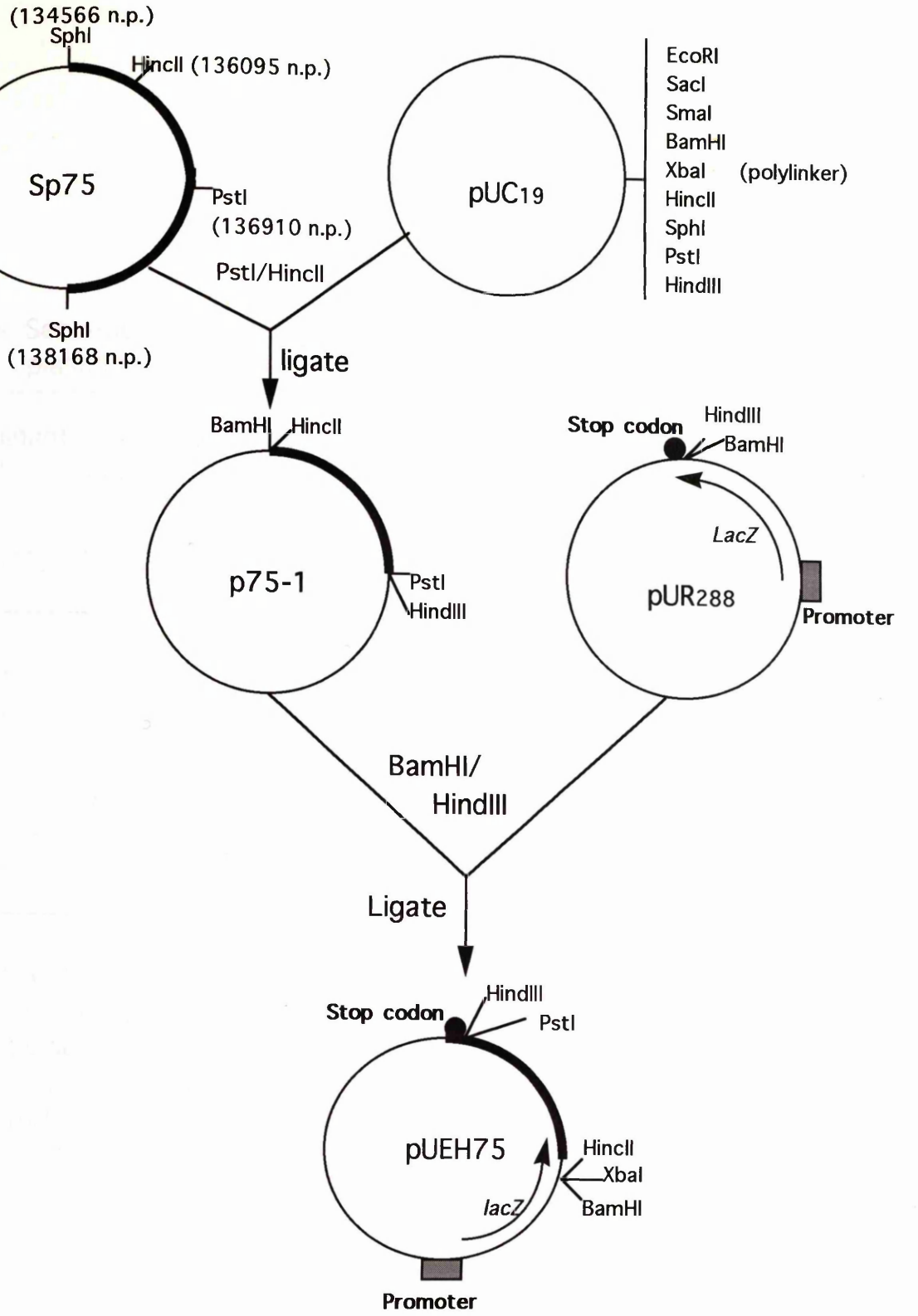
The sequence of junction between lacZ and insertion in pUEH71

pUR288 *LacZ* HindIII gene 71 encoding sequence

----GAA AGC TTT GCT GAT ACC-----
----CTT TCG AAA CGA CTA TGG-----

Figure 22. Generation of expression plasmid pUEH75.

The HincII/PstI fragment (136095-136910 n.p.) (thicker line) containing the coding sequence of amino acids 14-130 of gene 75 protein was cloned into the pUC19 HincII/PstI sites from plasmid p75, which contains the EHV1 SphI fragment (thicker line), to construct plasmid p75-1. The BamHI/HindIII fragment containing the HincII/PstI fragment in p75-1 was inserted into the pUR288 BamHI/HindIII sites to construct pUEH75. In pUEH75 the gene 75 coding sequence is fused in frame with the 3' end of the ß-galatosidase gene. The junction sequence between *lacZ* and the gene 75 protein encoding sequence is shown. The arrow indicates the junction and the insertion site (BamHI site) is shown in bold. The arrows show the orientation of the gene transcription.



The sequence of the junction between *lacZ* and insertion in pUEH75

pUR288 BamHI ▼ gene75 encoding sequence
---GG **GGA TCC** TCT AGA GTC AAC TAT---
---CC **CCT AGG** AGA TCT CAG TTG ATA---

Table 6. Sequence data of the constructed fusion protein expression plasmids*

recombinant plasmid	expressed gene	inserted fragment (n.p.)' 5'- 3'-end	encoded amino acid sequence	vector	insertion site
pUEH1	1	1329-1927	4- 202	pUR278	B/H"
pUEH2	2	2555-1184	2-205	pUR278	B/H"
pUEH67	67	125160-124411	12-260	pUR288	B/H"
pUEH71	71	130487-131487	464-797	pUR278	H"
pUEH75	75	136095-136910	14-130	pUR288	B/H"

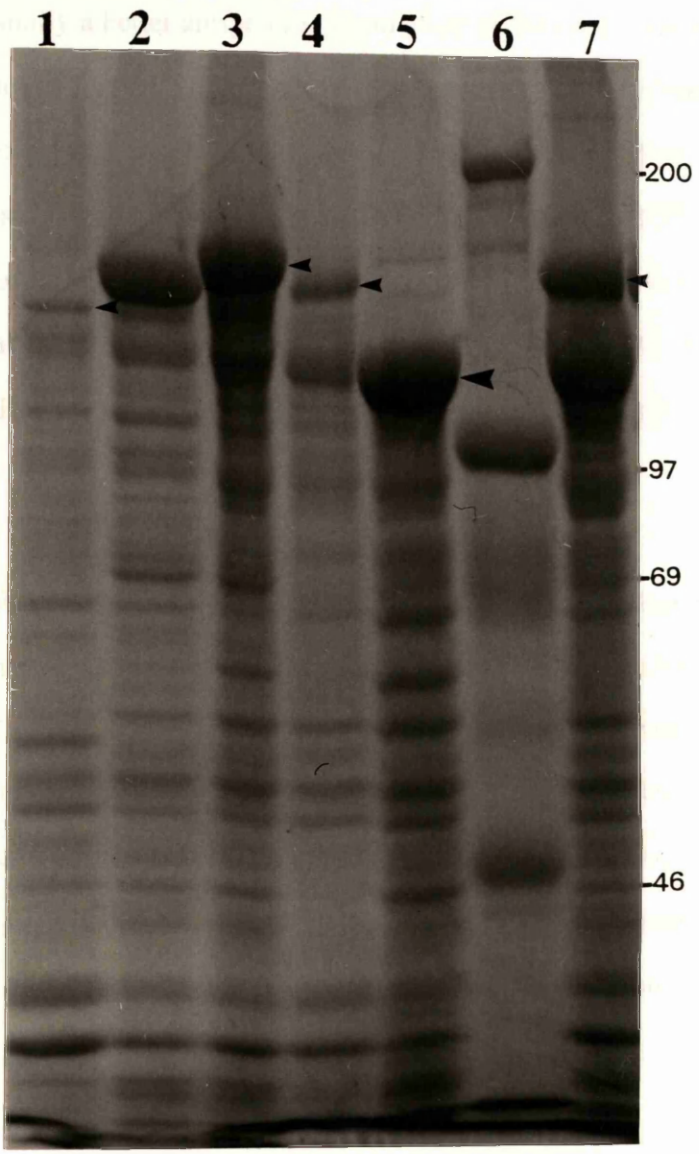
*The portion of the encoding sequences of the five target genes separately cloned into the pUR vector polycloning site to fuse in frame with the 3' end of the lacZ gene.

B/H": BamHI/HindIII; H": HindIII; n.p.': nucleotide position

of the gene 1 putative product and amino acids 2-205 of the EHV1 gene 2 putative product with M_rs of 154 KD and 159 KD respectively by 6% SDS-PAGE (Fig. 23, lanes 4 and 7). They were not present in extracts from pUR288 transformed cells (Fig. 23, lane, 5). However, a 116 KD ß-galactosidase band was also present as well as the higher M_r bands, which indicated that FP1 and FP2 were partially degraded after synthesis (Fig. 23, lanes 4 and 7). To prevent this degradation, different incubation temperatures (26°C, 31°C and 39°C) and different strains of host cells (*E.coli* NM522 and JM109) were employed, but degradation could not be prevented (data not shown). Fusion protein FP75 which consists of the N-terminus of ß-galactosidase fused with amino acids 14-130 of EHV1 gene 75 protein was expressed from pU75EH transformed cells as a protein with a M_r of 150 KD by 6% SDS-PAGE (Fig. 23, lane 1). It was not present in pUR288 transformed cell extracts (Fig. 23, lane 5). The 116 KD ß-galactosidase band was not present in pUEH75 transformed cell extracts. FP75 was poorly expressed in pUEH75 transformed cells. To increase the expression level, the transformed cells were incubated at different temperatures (27°C, 31°C) and induced with different amounts of IPTG. In addition, different strains of *E. Coli* , NM522 and JM109, were used as host cells to express the fusion protein, but the level of the FP75 expression could not be increased (data not shown). The fusion proteins FP67 and FP71, which consist of the N-terminus of ß-galactosidase fused with amino acids 12-260 of the EHV1 gene 67 protein and amino acids 464-797 of the EHV1 gene 71 protein respectively, were over expressed after induction with 1mM IPTG from pUEH67 and pUEH71 transformed cells as bands with apparent M_rs of 154 KD and 163 KD in 6% SDS-PAGE (Fig. 23, lanes 2 and 3), but were not present in pUR288 transformed cells (Fig. 23, lane 5). The 116 KD ß-galactosidase band were not present in extracts from pEH67 and pUEH71 transformed cells. Fractionation experiments showed that all the expressed fusion proteins were in the insoluble fractions as inclusion bodies (data not shown). These results showed that the five ß-galactosidase fusion proteins were expressed as insoluble proteins from recombinant cells with approximately the expected molecular weight.

Figure 23. Expression of five ß-galactosidase fusion proteins.

The XL1 cells transformed with recombinant plasmids, pUEH1, pUEH2, pUEH67, pUEH71 and pUEH75 were induced with 1mM IPTG after 2h incubation at 37°C and incubation continued for 2 h. The cells were then lysed and the polypeptides separated by 6% SDS-PAGE and stained with Coomassie blue. Lane 1, pUEH75 transformed cell extract; Lane 2, pUEH67 transformed cell extract; lane 3, pUEH71 transformed cell extract; Lane 4, pUEH1 transformed cell extract; Lane 5 pUR288 transformed cell extract; Lane 6 molecular weight marker; lane 7, pUEH2 transformed cell extract. Molecular weights are shown on the right hand side in KD. The fusion proteins are indicated by small arrows and the ß-galactosidase band by a large arrow.


1
2
3
4
5
6
7
200
97
69
46

3. 5. 3. Purification of the expressed fusion proteins and immunisation of rabbits to raise specific anti-sera.

There are several available methods to purify expressed proteins from *E. coli*. These produce different qualities and quantities of purified proteins. As our aim in purification of the expressed proteins was to use them as antigens to raise specific antibodies, and as an insoluble protein is usually a better antigen for stimulation of the host immune system than a soluble protein (Harlow, 1988), purification of the insoluble inclusion bodies, containing insoluble protein, from the lysed transformed cells was chosen as described in section (2.2.46). Using this method the fusion proteins FP1, FP2, FP67 FP71 and FP75 were purified from transformed cells as insoluble inclusion bodies. The amounts of purified insoluble protein (total protein) typically obtained from 100 ml of pU1EH, pU2EH, pU67EH, pU71EH and pU75EH transformed cells were 0.96 mg, 1.71 mg, 2.6 mg, 2.8 mg, and 0.71 mg, respectively.

To raise specific antibodies against the expressed gene products, two female New Zealand white rabbits were immunised with 0.9 mg/animal of the purified fusion protein, mixed with an equal volume of complete Freund's adjuvant, for the primary immunisation and 0.5 mg/animal of purified fusion protein, mixed with an equal volume of incomplete Freund's adjuvant, for the boost injections (see Section 2.2.47). After three boosts, the anti-sera were tested against EHV1 infected cell extracts by western blotting and immunoprecipitation. The results showed that two specific anti-sera, anti-67 and anti-71, were induced by FP67 and FP71, respectively. The results are presented in the next section.

Unfortunately, the other three fusion proteins did not induce any antisera to the gene poduct (data not shown). To examine the rabbit immune response to the fusion proteins the antisera were tested against the fusion proteins on Western blots. The results showed that all sera had a strong reaction to the appropriate purified ß-galactosidase fusion proteins (data not shown). To further determine whether there was a specific target protein band, the deletion mutants, ED1, ED2 and ED75, were used as negative controls to test antisera from FP1, FP2 and FP75

immunised rabbits. The results showed that the antisera did not detect any difference between wild-type and deletion mutants infected cell extracts by Western blot analysis (data not shown). This suggested that either no detectable specific antibodies against target gene products were induced by FP1, FP2 and FP75 or the level of virally expressed products of these genes are too low to detected by the antisera. Of significance is the fact that FP67 and 71 were expressed in the high yield.

3. 6. IDENTIFICATION AND CHARACTERISATION OF THE GENE 67 PRODUCT

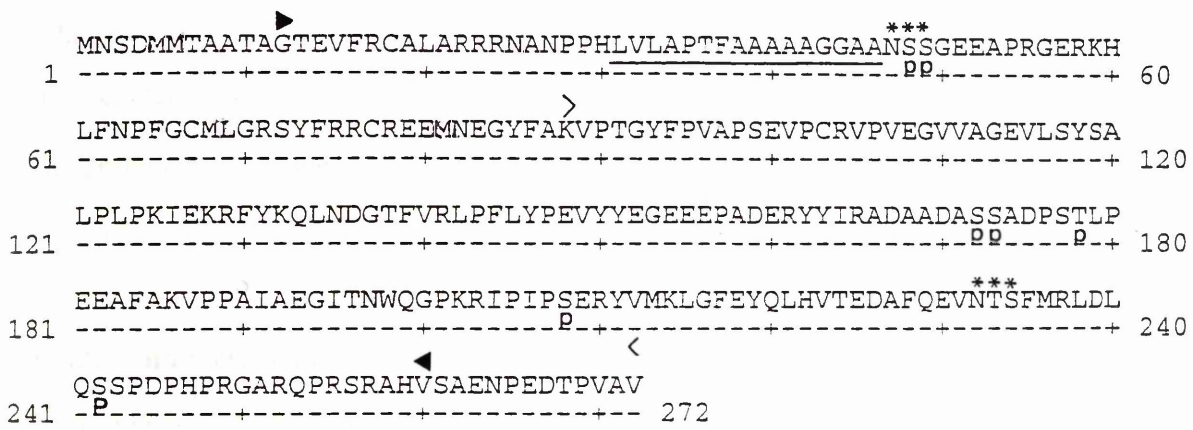
Sequence analysis showed that the putative gene 67 ORF in both repeat regions of the EHV1 genome encodes a putative 272 amino acids polypeptide with a M_r of 30 KD (Fig. 24) which at the time had no homologues in other sequenced herpesviruses. Recently a homologue has been identified in equine herpesvirus type 4 (Nagesha *et al.*, 1993) and bovine herpesvirus type 1 (Leung-Tack *et al.*, 1994). It has been reported that a mutation in gene 67 was the only alteration found in EHV1 strain RacH, an attenuated live vaccine (Mayr *et al.*, 1968; Osterrieder *et al.*, 1994a,b, 1995). The characterisation of ED67 in a mouse model also showed ED67 was attenuated (Tim Fitzmaurice personal conmunication). These features suggest that gene 67 may play a specific role in its natural host, particularly in determination of pathogenicity. To help elucidate the function of the gene, the product of gene 67 was identified and characterised.

3. 6. 1. Identification of the polypeptide product of EHV1 Ab4 gene 67.

An antiserum, anti-67, was produced against the fusion protein FP67. Western blotting experiments with EHV1 infected cell extracts showed that anti-67 at a dilution of 1:100-1:1000 recognised a 36 KD EHV1 infected cell (data not shown). To further confirm the specificity of anti-67 and to identify the gene 67 product, monolayers of BHK21/C13 cells

Figure 24. Predicted amino acid sequence of EHV1 gene 67 protein

The predicted gene 67 encoded protein amino acid sequence is shown. The underlined amino acid sequence indicates the hydrophobic region (Sun *et al.*, 1994b). The potential asparagine-linked glycosylation site is denoted by asterisks. The potential phosphorylation sites are indicated by **p** under the sequence. The numbers of amino acids are given on each side. The region (124416-124929 n.p.) removed in ED67 is limited by angle brackets. The sequence (124411-125160 n.p.) expressed from pUEH67 is limited by solid triangles.


MNSDMMTAATAGTEVFRCALARRRNANPPHLVLAPTFAAAAAGGAANSSGEEAPRGERKH
LFNPFGCMLGRSYFRRCREEMNEGYFAKVPTGYFPVAPSEVPCRVPVEGVVAGEVLSYSA
LPLPKIEKRFYKQLNDGTFVRLPFLYPEVYYEGEEEPADERYYIRADAADASSADPSTLP
EEAFAKVPPAIAEGITNWQGPKRIPIPSERYVMKLGFEYQLHVTEDAFQEVNTSFMRLDL
QSSPDPHPRGARQPRSRAHVSAENPEDTPVAV
1
60
61
120
121
180
181
240
241
272

were infected with EHV1, ED67 or Re67 and the polypeptides from the infected cells were separated by 10% SDS-PAGE and analysed by Western blotting with either anti-67 serum (dilution of 1:200) or preimmune serum (dilution of 1:80). The results showed that anti-67 specifically recognised an infected-cell polypeptide with apparent M_r of 36,000 D in a 10% SDS-PAGE in EHV1 and Re67 infected cell extracts (Fig. 25, a, lane 2 and b, lane 1); the polypeptide was not recognised in either mock infected cell extracts (Fig. 25a, lane 1 and b lane 2) or ED67 infected-cell extracts (Fig.25b, lane 3); it was also not recognised by preimmune serum (Fig. 25a, lane 3). The immunoreaction of anti-67 was inhibited by the FP67 fusion protein (Fig. 25a lane 5), but not by an unrelated fusion protein, FP71 (Fig. 25 a, lane 4). An additional infected-cell protein forming a weak band with M_r of 33,000 in 10% SDS-PAGE was also specifically detected by anti-67 (Fig. 25a, lane 2 and b, lane 1). The 36 KD polypeptide is absent in mutant ED67 infected cells and restored in Re67 infected cells confirming that the 36 KD polypeptide is encoded by EHV1 gene 67.

3.6.2. Characterisation of the EHV1 gene 67 protein product.

To further explore the properties of the gene 67 protein, the protein was characterised in terms of regulation of expression, localisation and post-translational modifications.

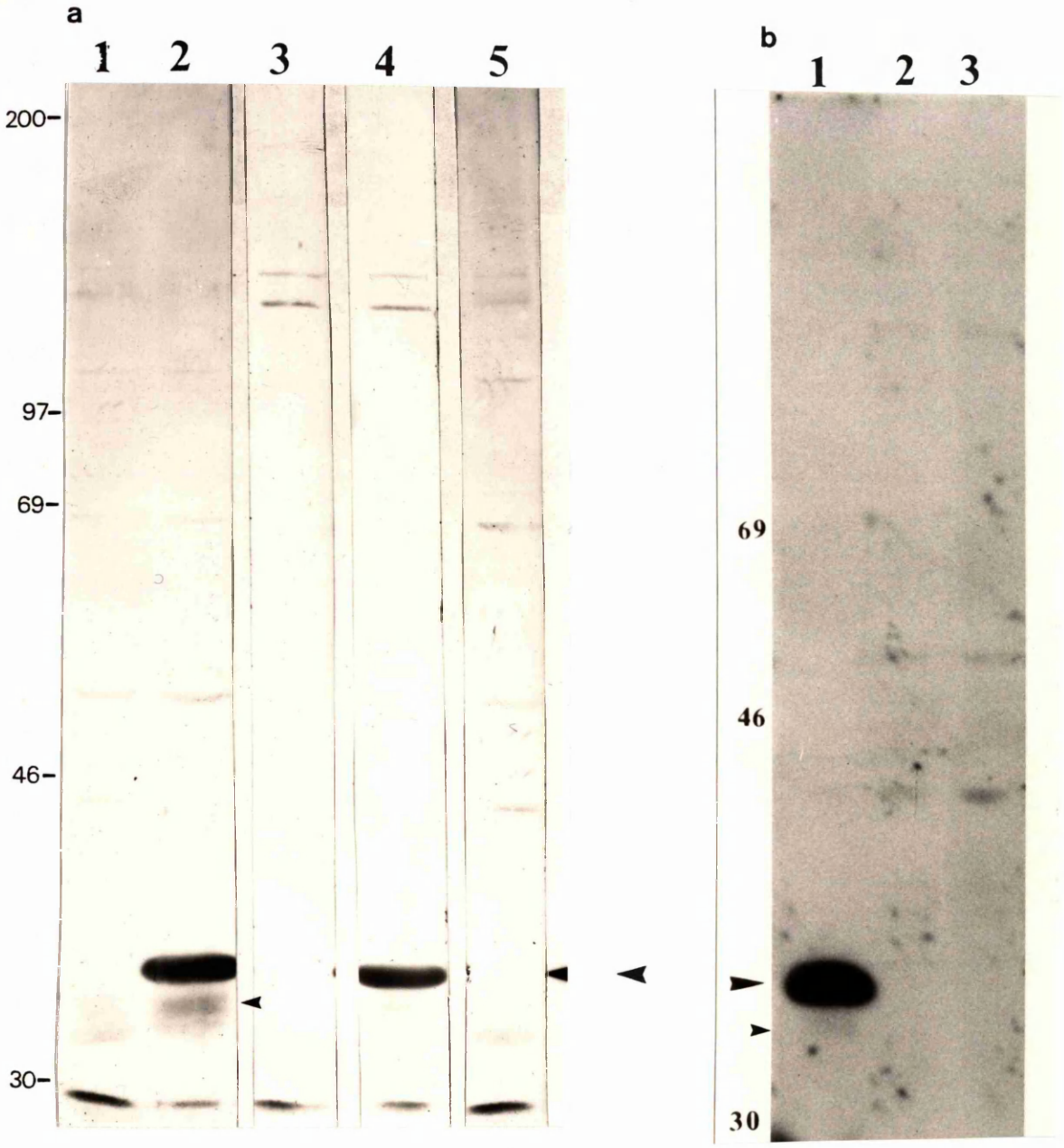
3.6.2.1. Regulation of expression of the gene 67 protein

EHV1 transcription and translation are regulated in a temporal pattern, i.e.. immediate early, early and late (IE, E, L) similar to that of HSV1 (Cohen *et al.,* 1975a; Gray *et al.*, 1987; Honess & Roizman, 1974; Clements *et al.*, 1977). To determine the kinetics of the gene 67 protein expression, a time course experiment was carried out in the presence or absence of phosphono acetic acid (PAA), an inhibitor of viral DNA replication. BHK21/C13 monolayers in 33 mm diameter petri dishes were infected with virus at 5 pfu/cell or mock infected in the presence or absence of 300 µg/ml of PAA. At various time points post infection, the cells were harvested into 150 µl of sample buffer. The polypeptides in the extracts were separated by 10% SDS-PAGE and analysed by Western blotting with anti-67. The results presented in Fig. 26 show that the 36 KD gene 67 product was first detected at 1 h

Figure 25. Western blot of electrophoretically separated EHV1 infected cell lysates probed with anti-67.

Lysates obtained from infected or mock infected cells at 20h post infection were separated by 10% SDS-PAGE and transferred to a nitrocellulose sheet which was divided and probed with anti-67 or preimmune serum. (a) Lane 1: mock infected; lanes 2, 3, 4, 5: EHV-1 Ab4 infected cell extracts; Lanes 1 and 2: probed by anti-67; Lane 3 probed by preimmune serum; Lane 4: probed by anti-67 in the presence of an unrelated fusion protein (20μg of FP71) ; Lane 5: probed by anti-67 serum in the presence of 20 μg of FP67. (b) Lane 1, Re67 infected cell extract; Lane 2, mock infected cell extract; Lane 3, ED67 infected cell extract, probed by anti-67.

Molecular weights are presented in KD on the left side. The 36 KD EHV1 gene 67 protein is indicated by a large arrow. The 33KD infected cell polypeptide is indicated by a small arrow.


a
1
2
3
4
5
200
97
69
46
30
b
1
2
3
69
46
30

p.i., with the amount gradually increasing until about 10 h p.i. and remaining constant up to 24 h p.i. In the presence of 300μg/ml PAA, expression of the 36 KD gene 67 polypeptide was slightly inhibited (2-4) fold (Fig. 26). At this concentration of PAA, EHV1 DNA replication is reduced to <5% of the level of the untreated control in BHK21/C13 cells (data not shown). This result indicated that virus DNA replication was not required for the expression of the 36KD polypeptide. For comparison, regulation of expression of a HSV1 early protein, $65K_{DBP}$, was examined under the same conditions. The results showed that the $65K_{DBP}$ was detected at 2h p.i. and in the presence of PAA, expression of the $65K_{DBP}$ was similarly only slightly inhibited (data not shown). Finally, as the 36 KD polypeptide was expressed at a very early stage of virus infection (1h p.i.), it was necessary to determine whether the 36KD polypeptide is regulated as an immediate early protein. To do this EHV1 immediate early proteins were prepared as described in Section 2.2.23 and subjected to Western blotting with anti-67 serum. The results presented in Fig.27 showed that the 36 KD polypeptide was not detected among EHV1 immediate early polypeptides (Fig. 27 a, lane 3), but it was present in untreated control (Fig. 27 lane 1). As a comparison, using the same conditions HSV1 immediate early proteins were prepared, and the HSV1 IE polypeptide, Vmw110 (Stow *et al.*, 1986; Sacks & Schaffer, 1987) was clearly detected in both immediate early and total infected cell extracts (Fig 27 b lanes 3 and 1). These results confirmed that the 36 KD gene 67 product was not expressed as an immediate early polypeptide. Overall these results indicate that the 36KD gene 67 product is regulated as an early protein in EHV1 infected cells.

It should be noted that four additional species with M_rs of 33KD, 31.5KD, 29KD and 26KD were also detected along with the 36 KD polypeptide by anti-67 in EHV1 infected cells (Fig. 26). The 26 KD polypeptide was initially expressed at the same time as the 36 KD protein , but the amount gradually decreased. The 33KD polypeptide was expressed as a less abundant polypeptide with the same kinetics as the 36 KD band, appearing at 1 h p.i. and gradually increased until 12 h p.i. Two other polypeptides (31.5 and 29 KD) appeared at 12 h p.i. and gradually decreasing until 24 h p.i. These bands were not present in mock infected extracts

Figure 26. Time course of EHV-1 gene 67 expression.

BHK21/C13 cells were infected with 5 pfu/cell EHV1 or mock infected and harvested at various times up to 24h (-0.3 h equal to 33 min after adding virus into cells; 0 h equal to 1 h post adsorption; 1, 2, 4, 6, 8, 10, 12, 14, 18, 24 h time post adsorption) post infection. To determine if the gene 67 protein is a true late protein, proteins were also extracted at 6, 8, 12, 16 and 18h p.i. from BHK21/C13 cells infected with EHV-1 in the presence of 300 μg/ml PAA. The infected cell polypeptides were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Expression of the gene 67 protein was analysed by Western blotting with anti-67. M_r are indicated as KD on the left. The gene 67 protein product is indicated by a large arrow on the right hand side. The four other detected polypeptides are indicated by small arrows on the right hand side.

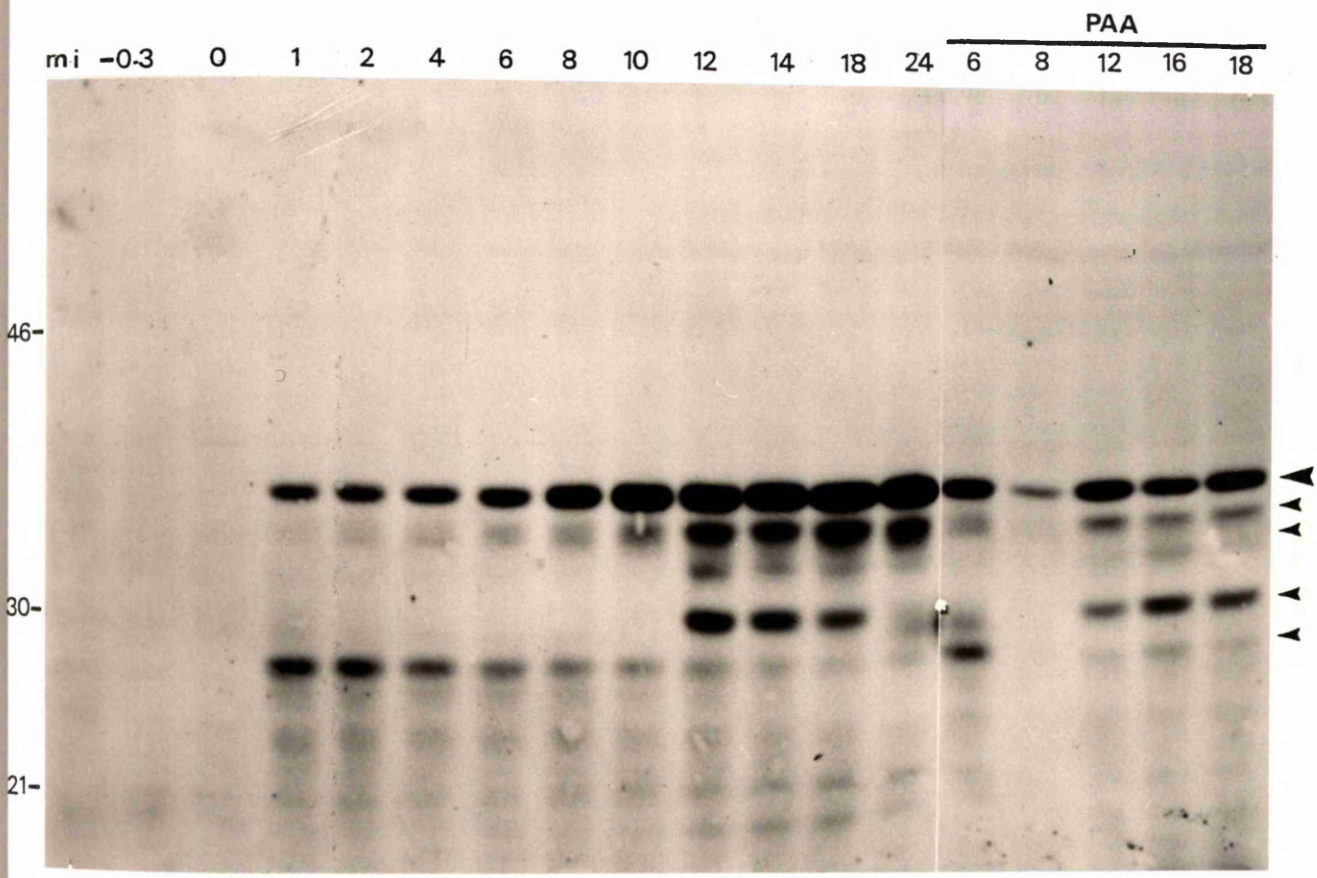
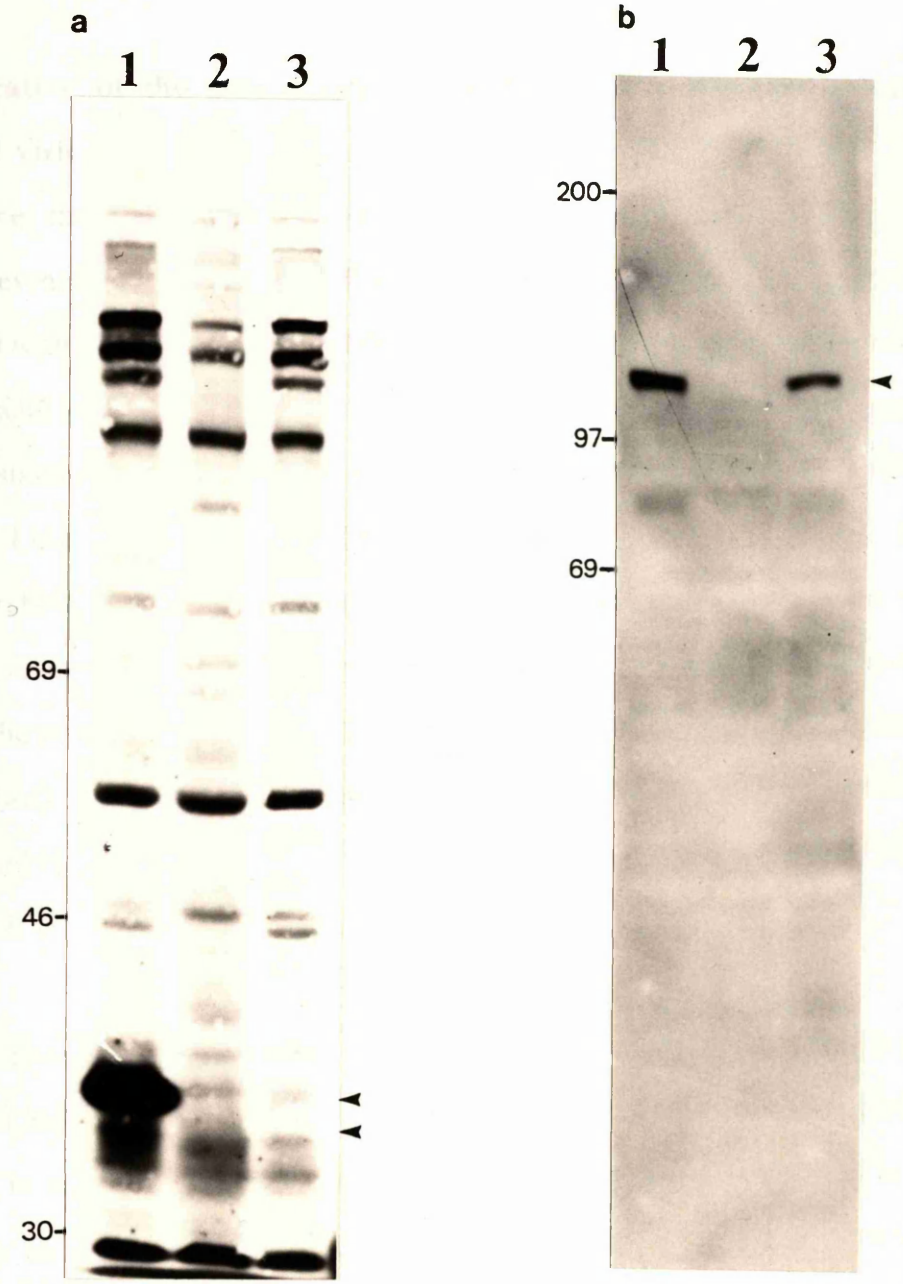

PAA
mi -0.3 0 1 2 4 6 8 10 12 14 18 24 6 8 12 16 18
46-
30-
21-

Figure 27. Western blot of (a) EHV1 and (b) HSV1 induced immediate early polypeptides.

a) Probed with anti-67; b) Probed by anti-HSV-1 VmwIE110.

Lane 1 untreated 24h infected cell extracts; Lane 2: mock infected extracts; Lane 3: infected cell polypeptides prepared under immediate-early conditions; Molecular weight markers are shown on the left in KD. The 36 KD and 33 KD proteins and HSV1 Vmw110 are indicated by arrows.


a
1
2
3
69
46
30
b
1
2
3
200
97
69

(Fig. 26, lane 1) nor in ED67 infected cell extracts (Fig. 25, b, lane 3), indicating that they are infected cell polypeptides and probably relate to gene 67. A pulse-chase experiment showed that the 36 KD gene protein could not be chased into any other species even after a 3 h chase (data not shown). Thus the relationship of these proteins to each other is unclear.

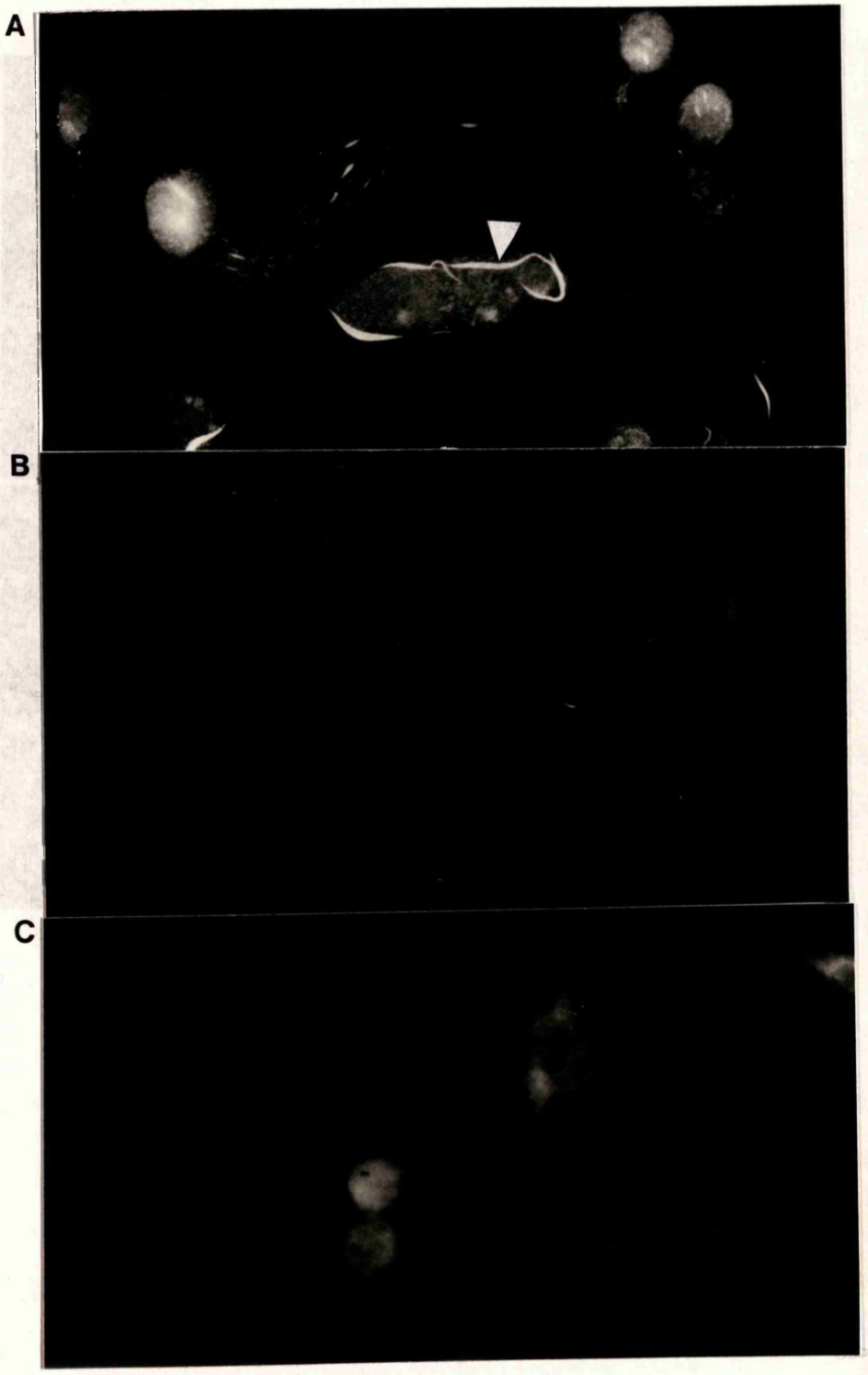
3.6.2.2. Location of the gene 67-encoded polypeptide within infected cells and virions.

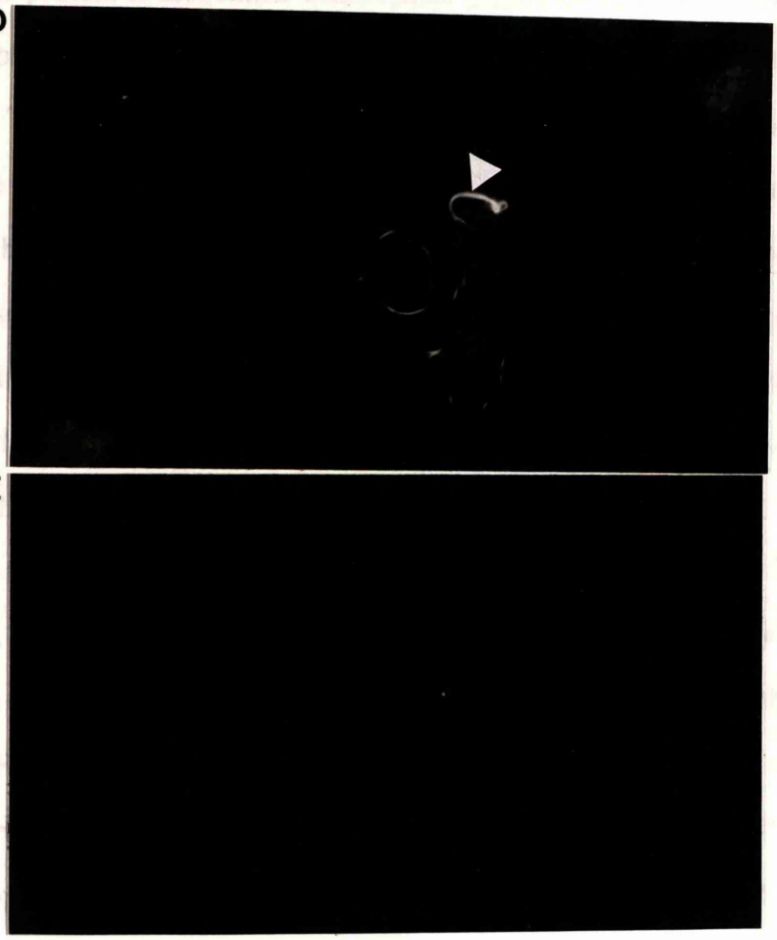
To determine the subcellular distribution of the gene 67 polypeptide, indirect immunofluoresence was used. 50% confluent monolayers of BHK21/C13 cells or Vero cells were infected with virus at 0.1 pfu/cell and fixed at 16h p.i. For surface staining, infected cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature; for intracellular staining, the cells were fixed with 4% paraformaldehyde and permeabilised by 1% NP40 for 1 h at room temperature. The cells were stained with anti-67 (dilution of 1:100) and an anti-rabbit antibody fluorescent conjugate. The results showed that the gene 67 product is present in the cytoplasm of infected cells as a ribbon or filament like structure in the cytoplasm (Fig. 28 a). No staining was detected in non-permeabilized cells (data not shown). The structures were not detected in mock infected or ED67 infected cells (Fig. 28 b, and c). The distribution of the gene 67 protein in BHK21/C13 cells (Fig. 28 a) and Vero cells is indistinguishable (Fig. 28 d).

To further localise the gene 67 product, BHK21/C13 cells were infected either in the presence or absence of Nocodazole or Brefeldin A. Nocodazole, a synthetic microtubule inhibitor has been shown to cause the disappearance of mictobules from mammalian cells in culture. Brefeldin A, an inhibitor of transport between the ER and the Golgi apparatus which ultimately leads to dissolution of Golgi cisternae has been found to disrupt the movement of newly synthesised membrane proteins into the Golgi apparatus (Doms *et al.*, 1989; Harris *et al.*, 1963; Lippincott-Schwartz *et al.*, 1989). At 20 h p.i., the treated cells were fixed and stained with anti-67. The results showed that neither inhibitor affected the distribution of the gene 67 product in the cytoplasm of infected cells (data not shown). In addition, infected

Figure. 28. Indirect immunofluoresence detection of the gene 67 protein product within cells by anti-67.

Infected or mock infected cells were fixed with 4% paraformaldehyde and treated with 1% NP40 at 16 h p.i. The cells were then analysed by indirect immunofluorescence with anti-67. **(a)** EHV1 infected BHK21/C13 cells; **(b)** mock infected cells; **(c)** ED67 infected BHK21/C13 cells. **(d)** EHV1 infected Vero cells. **(e)** Mock infected Vero cells. magnification, x40. Arrowheads, filament-like strain.


A
B
C


D
E

cells were fixed and stained either with anti-tubulin or anti-actin antibodies plus anti-67 or with anti-67 alone. Double staining showed that the gene 67 product did not colocalise with tubulin or actin (data not shown).

Amino acid analysis of the gene 67 protein showed that the protein contains a N-terminal hydrophobic region (Fig. 24) which could serve as a transmembrane domain. To determine whether the protein is associated with cellular membranes, infected cells were fractionated into a soluble cytosol fraction and a membrane-containing fraction using the method described in 2.2.24. The membrane fraction was further separated into high affinity and low affinity membrane bound protein fractions by the addition of 1M -NaCl and 100mM-sodium carbonate buffer (pH 11.5), which converts closed membrane vesicles to open membrane sheets subsequently releasing contents and peripheral membrane proteins into the supernatant (low affinity membrane proteins). The polypeptides from the fractions were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane and Western blotted with anti-67 serum. The results showed that most of the gene 67 protein was stably bound to the membrane fraction in the presence of 1M -NaCl and 100mM-sodium carbonate, pH 11.5 (Fig 29 lane 3). A small proportion of the gene 67 protein was also detected in the low affinity membrane fraction and the cytosol (Fig. 29, lanes 4 and 5), but not in the supernatant of the medium (Fig. 29, lane 6). This result suggests that the gene 67 protein is associated with cellular membranes. Together these results suggested that the gene 67 protein was distributed in the cytoplasm as a ribbon-like structure, associated with intracellular membranes, but not colocalized with microtubule, Golgi, tubulin or actin.

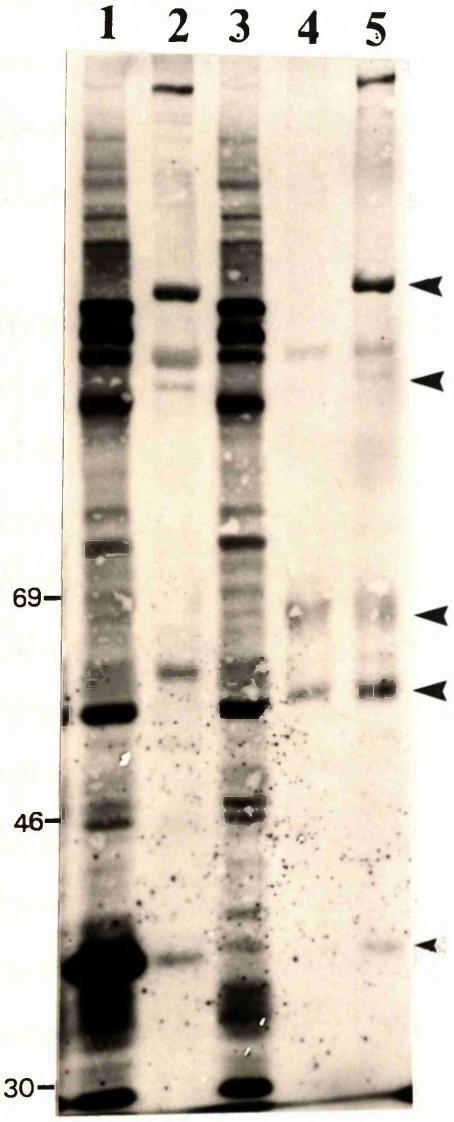
To determine whether the 36 KD polypeptide is a component of virions, virion polypeptides were extracted from purified virion particles with sample buffer and separated by 10% SDS-PAGE. The polypeptides were then transferred to a nitrocellulose membrane and immunoblotted with anti-67 serum. For further localisation of the gene 67 polypeptide within virions, purified virions were treated with 5% NP40 and separated into insoluble nucleocapsid/tegument and soluble envelope/tegument fractions by centrifugation (12,000 r.

Figure 29. Western blot of EHV1 infected subcellular fractions.
BHK21/C13 cells were infected with EHV-1 at 5 pfu/cell and at 16h p.i. the infected cells were separated into membrane and cytosol fractions. The membrane fraction was further separated into high and low affinity membrane protein fractions using 1M NaCl and sodium carbonate, pH11.5. The polypeptides in the fractions were analysed by Western blotting with anti-67. Lane 1: mock infected cell extracts; Lane 2: total cellular extract; Lane 3: membrane proteins stable in 1M NaCl and sodium carbonate, pH11.5; lane 4: low affinity membrane proteins stable in only 150mM NaCl; Lane 5: cytosol fraction; Lane 6: infected cell medium. The 36 KD EHV1 gene 67 protein is indicated by a large arrow. The 33 KD polypeptide is marked by a smaller arrow.


1 2 3 4 5 6
69
46
30

Figure 30. Western blot of EHV1 virion fractions with anti-67.

Purified EHV-1 virions were treated with 0.5% NP40 and separated into soluble and insoluble fractions by centrifugation. The fractions were then Western blotted with anti-67. Lane 1, infected cell extract; Lane 2, purified virions; Lane 3, mock infected cell extracts; Lane 4 supernatant of virions treated with NP40; and Lane 5, insoluble pellet from treated virions. Mr are indicated as KD on the left. The 36KD gene 67 product is indicated by a small arrow and four other virion background polypeptides by large arrows.


1
2
3
4
5
69
46
30

p.m. for 2 h). The polypeptides extracted from the two fractions were separated by SDS-PAGE and Western blotted with anti-67. The results presented in Fig. 30 showed that the 36KD polypeptide was detected in purified virions (lane 2) and in the tegument/nucleocapsid fraction (lane 5).In compared with its high expression in infected cells, only low abundance of the protein incorperated into virions. The anti-67 serum also recognised several other higher M_r virion nucleocapsid/tegument polypeptides with apparent M_r of 145KD, 120KD, 65KD and 55KD, which were not detected in mock infected (Lane 3) or infected cell extracts (Lane 1). However, the crossreaction between them on molecular level is unclear.

3.6.2.3. Transient expression of the gene 67 product in BHK21/C13 cells.

To further analyse the gene 67 polypeptide, an expression plasmid, pCMV67, was constructed. The gene 67 ORF was amplified by PCR using two primers containing a BamHI site. The amplified fragment was digested by BamHI and cloned into the BamHI site of pCMV10 under the CMV immediate early promoter (Fig. 31). To transiently express the gene 67 protein, 50% confluent monolayers of BHK21/C13 cells were lipofected with pCMV67 or control pCMV10 as discribed in section 2.2.32. At 30 h post lipofecton, the cells were either fixed and subjected to indirect immunofluoresence or lysed with sample buffer and subjected to Western blotting with anti-67 serum. A Western blotting experiment of cell extracts showed that the 36 KD gene 67 polypeptide and the 33KD protein were detected by anti-67 serum in pCMV67 lipofected cell extracts (Fig. 32 lane, 3), but not in mock infected (Fig. 32, lane 2) and pCMV10 lipofected cells extracts (Fig. 32, lane 4). Indirect immunofluorescence experiments with anti-67 revealed that the transiently expressed gene 67 protein was distributed within the cytoplasm coalescing immediately adjacent to the nucleus such that a solid band structure surrounded the nucleus (Fig. 33, a). No specific staining was detected in pCMV10 lipofected cells (Fig 33, b). This result shows that the protein expressed from the cloned gene 67 ORF in BHK21 cells was also recognised by anti-67 serum and had the same M_r and distribution pattern as that expressed in EHV1 infected cells (Fig. 32, lane 1). This result further confirmed that the 36 KD polypeptide recognised by anti-67 was encoded by EHV1 gene 67. In addition, it also indicates that the filament or ribbon-like distribution of

Figure 31. Construction of expression plasmid pCMV67.

The gene 67 ORF (124379-125196 n.p.) was amplified by PCR using primers:

(1),5'-T ACT AGATCT **ATG** AAC TCC GAT TA-3'

(2),5'-T ATC AGATCT CAG AGA TAC AGA TTA-3', containing BamHI sites (underlined), from Sp67. The amplified 817 bp fragment was digested with BamHI and cloned into the pCMV10 BamHI site under the CMV immediate early promoter in the correct orientation to construct pCMV67. Arrows show the orientation of gene 67 transcription. The initiating codon (ATG) of gene 67 is shown in bold in primer (1).

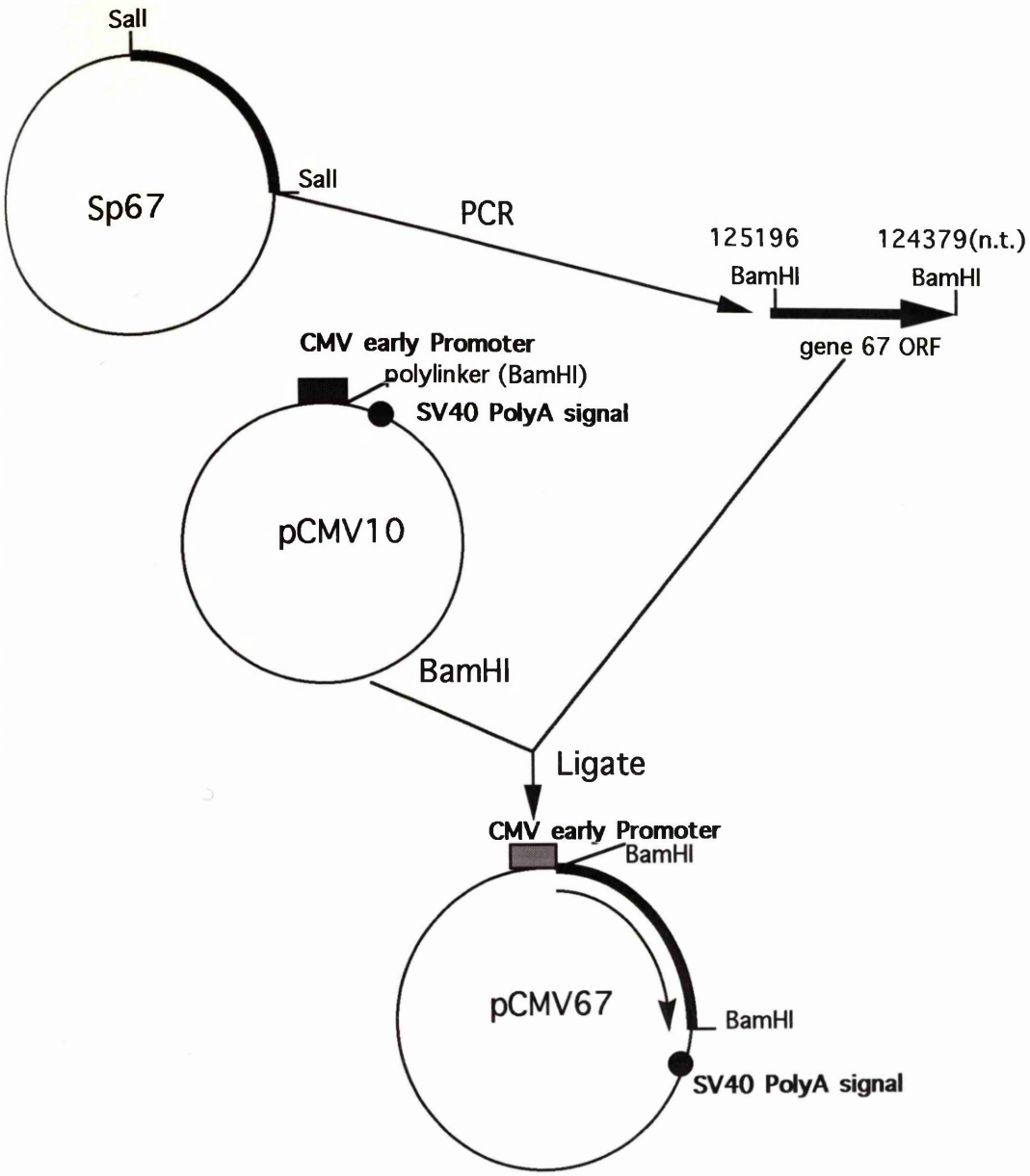

SalI
Sp67
SalI
PCR
125196
BamHI
124379(n.t.)
BamHI
gene 67 ORF
CMV early Promoter
polylinker (BamHI)
SV40 PolyA signal
pCMV10
BamHI
Ligate
CMV early Promoter
BamHI
pCMV67
BamHI
SV40 PolyA signal

Figure. 32. Lipofected BHK21/C13 cell extracts analysed by Western blotting.

BHK21/C13 cells were either lipofected with plasmid DNA, infected with EHV1 or mock infected. Infected cells were harvested at 16h p.i. and lipofected cells were harvested at 30h post lipofection. The cell extracts were analysed by Western blotting with anti-67 serum. Lane 1: EHV1 infected cell extract; Lane 2: mock infected cell extract; Lane 3: pCMV67 lipofected cell extract and lane 4: pCMV10 lipofected cell extract. Molecular weight markers are indicated as KD on the left. The 36 KD gene 67 product by a large arrow and the 33 KD protein by a small arrow on the right.

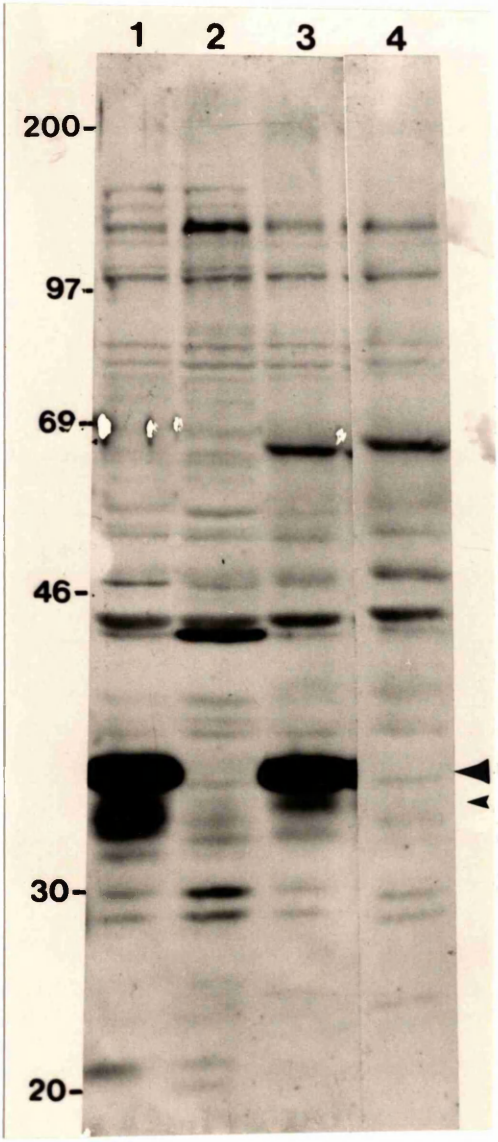
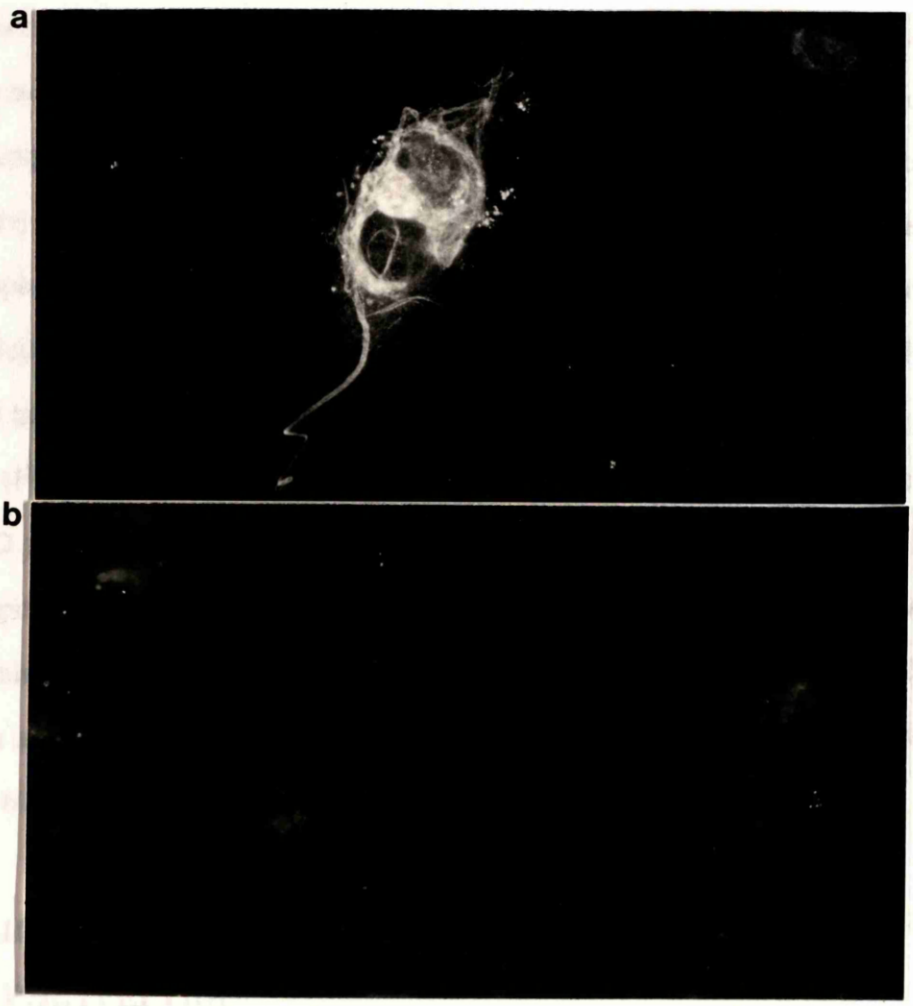

1
2
3
4
200-
97-
69-
46-
30-
20-

Figure 33. Indirect immunofluorescence of lipofected BHK21 cells.
BKH21/C13 cells were lipofected with pCMV67 (A) or pCMV10 (B). The cells were fixed at 30 h post lipofection and analysed by indirect immunofluoresence with anti-67.


a
b

the gene 67 protein is independent of other EHV1 gene products.

3.6.2.4. Post-translational modification of the gene 67 polypeptide.

Sequence analysis suggested that the gene 67 product contained two putative N-linked glycosylation sites and six putative phosphorylation sites. To determine whether the gene 67 protein is a glycoprotein or a phosphoprotein, infected cells were either labelled with ^{35}S methionine, ^{14}C glucosamine or ^{32}P orthophosphate or treated by glycosylation inhibitors (tunicamycin or monensin). Infected cells were lysed at 20 h p.i. and subjected to immunoprecipitation with anti-67 serum. Immunoprecipitation experiments demonstrated that the 36KD polypeptide is labelled both by ^{35}S methionine (Fig. 34 a lane 1) and by ^{32}P orthophosphate (Fig. 34 b lane 4), but not by ^{14}C glucosamine (data not shown). The ^{32}P labelled 36 KD polypeptide was not precipitated from mock infected cells (Fig. 34 a lane 2 and b lane 5). The preimmune serum did not precipitate the polypeptide from EHV1 infected cells (Fig. 34 a lane 3 and b lane 6). The fusion protein FP67 inhibited precipitation of the 36KD polypeptide (Fig. 34b lane 3), but an irrelevant fusion protein did not inhibit the precipitation (Fig. 34 b, lane 2). Treatment with glycosylation inhibitors showed that neither tunicamycin nor monensin, altered the mobility of the gene 67 product on 10% SDS-PAGE when analysed by Western blotting (data not shown). These results demonstrated that the gene 67 product is a phosphoprotein, but not a glycoprotein.

3.7. IDENTIFICATION AND CHARACTERISATION OF THE GENE 71 POLYPEPTIDE

Sequence analysis predicted that EHV1 gene 71 encodes a 797 amino acids polypeptide. The predicted polypeptide contains amino- and carboxy-terminal hydrophobic domains which may serve as a potential signal sequence and transmembrane domain. It also contains a single N-linked glycosylation site and a serine/threonine rich region which is likely to serve as a O-linked glycosylation site (Fig. 35). These features suggested that gene 71 could encode a class I membrane glycoprotein with heavy O-linked glycosylation. To elucidate the function of the gene and to confirm the predicted features of the gene 71 protein, a specific anti-serum,

Figure. 34. (a). Autoradiography of immunoprecipitated ^{35}S labelled gene 67 polypeptide

Extracted proteins from cells labelled with [^{35}S] methionine were immunoprecipitated with either anti-67 serum or preimmune serum and analysed by 10% SDS-PAGE. Lane 1: EHV1 infected cell extract precipitated by anti-67; Lane 2: mock-infected cell extract precipitated by anti-67; Lane 3: EHV-1 infected cell extract precipitated by preimmune serum. The gene 67 36 KD product is indicated by a large arrow on the left hand side. The specific 33 KD and 31.5 KD bands are marked by small arrows on the left hand side. M_r are indicated as KD on the left.

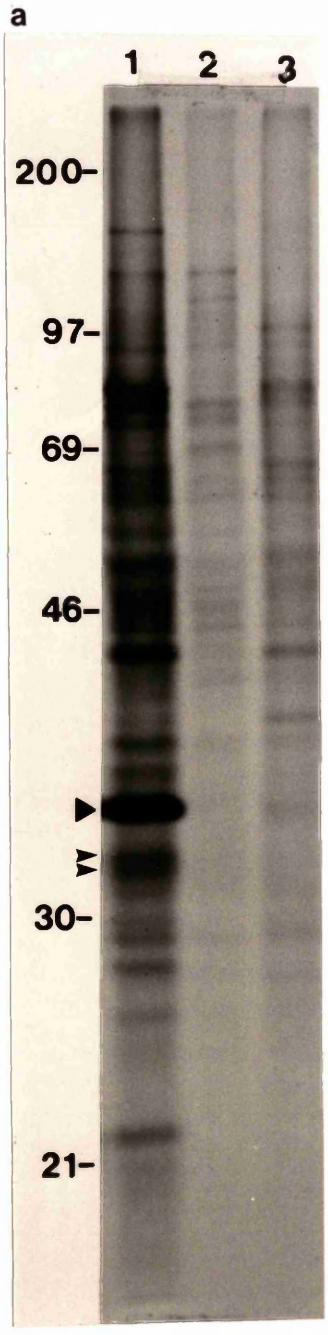

a
1
2
3
200-
97-
69-
46-
30-
21-

Figure 34 (b). Autoradiography of immunoprecipitated ^{32}P labelled gene 67 protein.

Extracted proteins from cells labelled with [^{32}P] orthophosphate, immunoprecipitated with anti-67 or preimmune serum and analysed by 10% SDS-PAGE. Lane 1: infected cell extract; Lanes 2-4 infected cell extracts immunoprecipitated by anti-67; Lane 2: in the presence of 20μg unrelated fusion protein; Lane 3: in the presence of 20μg pUEX67 fusion protein; lane 4: with anti-67 alone; Lane 5: mock infected cell extracts precipitated by anti-67 and Lane 6: EHV-1 infected cell extract precipitated with preimmune serum. Molecular weight markers are indicated on the left in KD and the 36 KD gene 67 product by an arrow on the right.

b

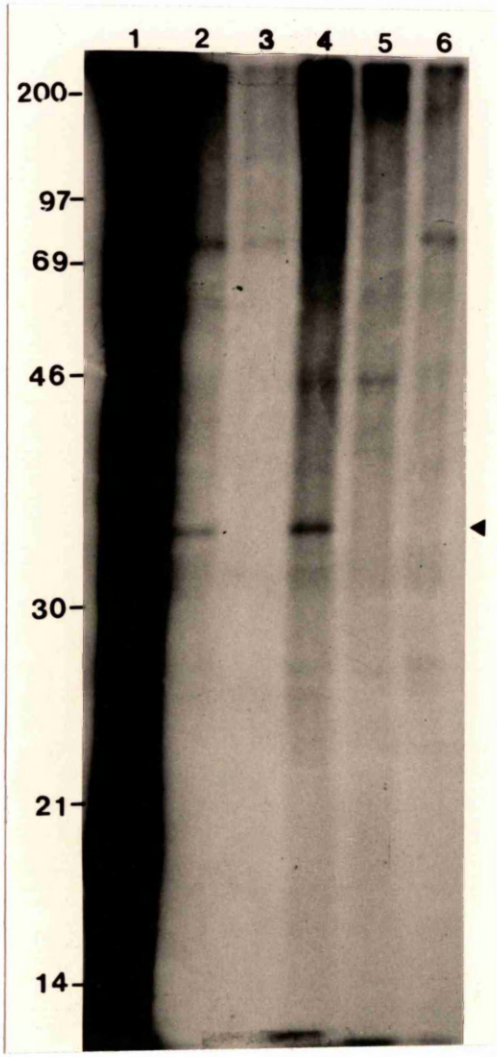
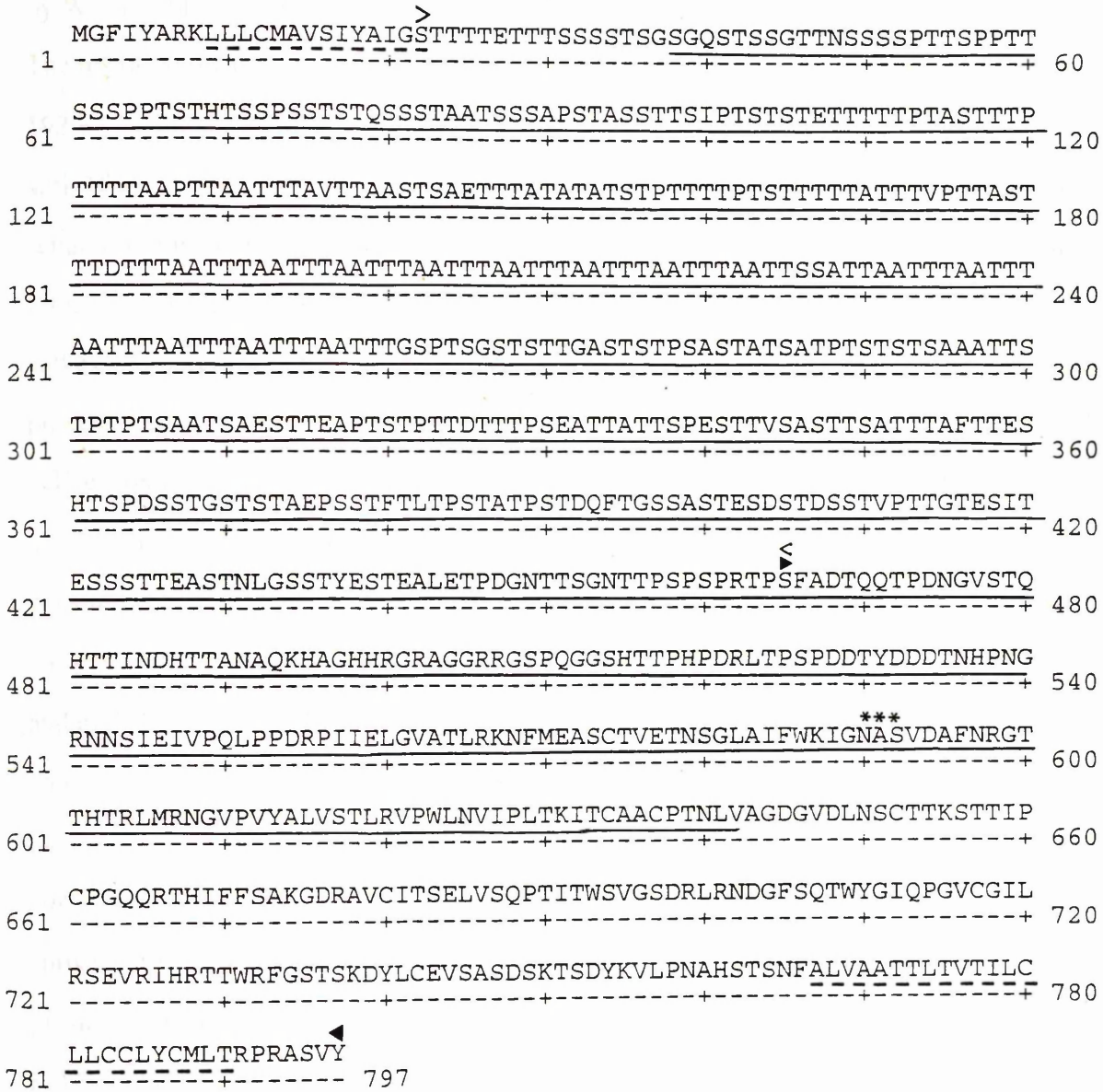


Figure. 35. Predicted amino acid sequence of the EHV-1 gene 71 protein.

The amino acid sequence of the EHV1 Ab4 gene 71 protein was predicted from analysis of DNA sequence data. Hydrophobic amino- and carboxy-terminal domains are underlined (----). A single potential N-linked glycosylation site is denoted by asterisks. The domain which is rich in serine and threonine residues (potential O-linked glycosylation sites) is delimited by angle brackets. The expressed domain in the fusion protein PUEH71 is delimited by solid triangles. The domain which was deleted in the mutant ED71 is underlined with a solid line. Residue numbers are given on each side.


MGFIYARKLLLCMAVSIYAIGSTTTTETTTSSSSTSGSGQSTSSGTTNSSSSPTTSPPTT
SSSPPTSTHTSSPSSTSTQSSSTAATSSSAPSTASSTTSIPTSTSTETTTTTPTASTTTP
TTTTAAPTTAATTTAVTTAASTSAETTTATATATSTPTTTTPTSTTTTTATTTVPTTAST
TTDTTTAATTTAATTTAATTTAATTTAATTTAATTTAATTTAATTSSATTAATTTAATTT
AATTTAATTTAATTTAATTTGSPTSGSTSTTGASTSTPSASTATSATPTSTSTSAAATTS
TPTPTSAATSAESTTEAPTSTPTTDTTTPSEATTATTSPESTTVSASTTSATTTAFTTES
HTSPDSSTGSTSTAEPSSTFTLTPSTATPSTDQFTGSSASTESDSTDSSTVPTTGTESIT
ESSSTTEASTNLGSSTYESTEALETPDGNTTSGNTTPSPSPRTPSFADTQQTPDNGVSTQ
HTTINDHTTANAQKHAGHHRGRAGGRRGSPQGGSHTTPHPDRLTPSPDDTYDDDTNHPNG
RNNSIEIVPQLPPDRPIIELGVATLRKNFMEASCTVETNSGLAIFWKIGNASVDAFNRGT
THTRLMRNGVPVYALVSTLRVPWLNVIPLTKITCAACPTNLVAGDGVDLNSCTTKSTTIP
CPGQQRTHIFFSAKGDRAVCITSELVSQPTITWSVGSDRLRNDGFSQTWYGIQPGVCGIL
RSEVRIHRTTWRFGSTSKDYLCEVSASDSKTSDYKVLPNAHSTSNFALVAATTLTVTILC
LLCCLYCMLTRPRASVY
797

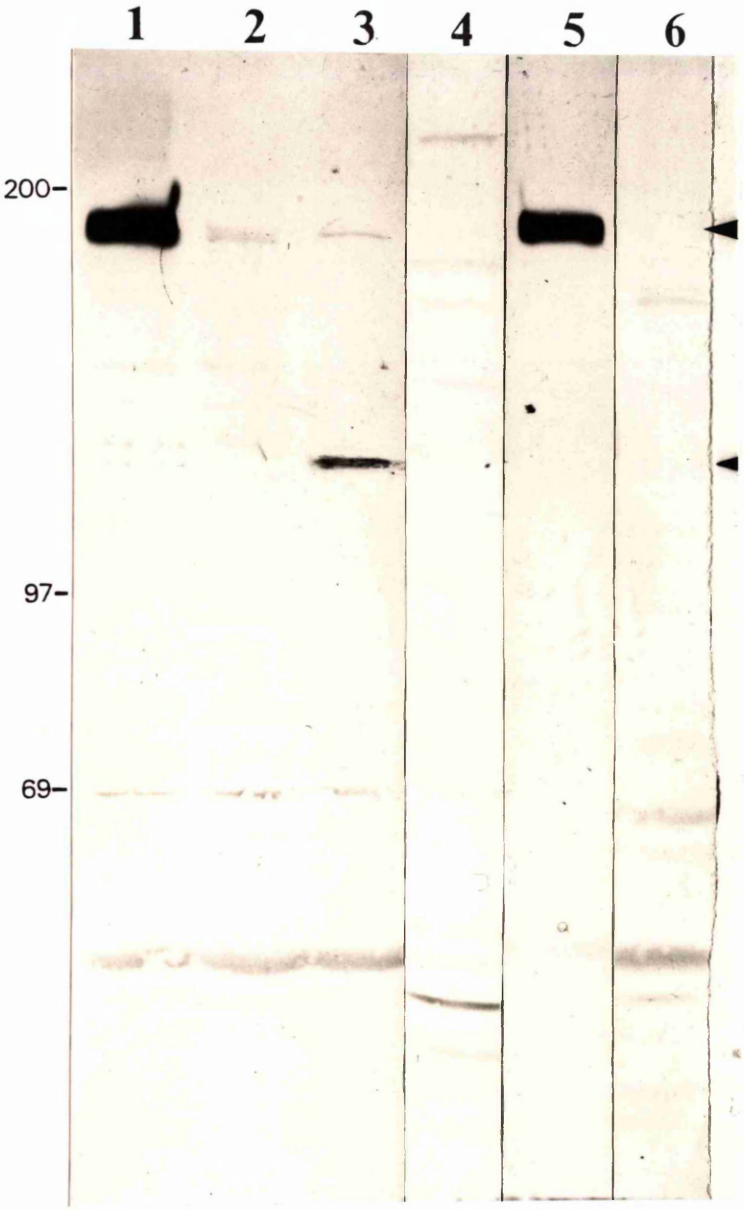
anti-71 was used to identify and characterise the protein product of gene 71.

3.7.1. Identification of the EHV1 gene 71 protein product

To identify the gene 71 protein product, a specific antiserum, anti-71, was raised against a carboxy-terminal portion of gene 71/ß-galactosidase fusion protein, FP71. Anti-71 was tested by Western blotting against EHV1 infected cell extracts and pU71HE transformed cell lysates. The results show that anti-71 (1:200) could recognise an infected cell polypeptide with a M_r of 192 KD and the FP71 fusion protein (data not shown). To further determine the specificity of anti-71 and subsequently identify the gene 71 product, monolayers of BHK21/C13 cells were separately infected with 5 pfu/cell of EHV1, ED71 and Re71, a revertant of ED71, or mock infected and the cells were harvested into sample buffer at 20 h p.i. The polypeptides in the cell extracts were separated by 8% SDS-PAGE and Western blotted with anti-71. The results presented in Fig. 36 show that anti-71 specifically recognises a polypeptide in EHV1 infected cell extracts which is present as a broad band at a position corresponding to a M_r of approximately 192 KD on 8% SDS-PAGE (Fig. 36, lane 1). The polypeptide was not detected in mock-infected cell extracts by anti-71 (Fig. 36, lane 2) and was not recognised by the preimmune serum (Fig. 36, lane 4). The immunoreaction between the gene 71 product and anti-71 is specifically inhibited by the gene 71 fusion protein (Fig. 36, lane 6), but not by an unrelated fusion protein (Fig. 36, lane 5). The 192 KD polypeptide was not detected from the deletion mutant ED71 infected cell extracts by anti-71 (Fig. 36, lane 3). However, a band corresponding to a M_r of 116KD was detected by anti-71(Fig. 36, lane 3). Since the antiserum was raised against a ß-galactosidase fusion protein, this 116 KD polypeptide was presumed to correspond to ß-galactosidase (116 KD) produced by the *lac* Z gene inserted in ED71. This was confirmed by a commercial ß-galactosidase monoclonal antibody which also recognised this 116 KD polypeptide (data not shown). These results demonstrate that gene 71 encodes a polypeptide with an apparent M_r of 192 KD in EHV1 Ab4 infected cells.

Figure. 36. Western blot of electrophoretically separated cell lysates

Lysates obtained from infected or mock infected cells at 20h post infection were separated on 8% SDS-PAGE and transferred to nitrocellulose and probed with antisera. Lane 2, mock-infected cell extract probed by anti-71 (1:100). Lanes 1,4,5 and 6, EHV-1 infected cell extracts: probed with anti-71 alone (lane 1), preimmune serum (lane 4), anti-71 with 20µg of an unrelated fusion protein (lane 5), anti-71 in the presence of 20µg of pUEH71 fusion protein (lane 6); Lane 3, ED71 mutant infected cell extract probed by anti-71 serum. The upper arrow marks the 192 KD protein of gene 71. The lower arrow indicates the position of the ß-galactosidase band. Molecular weight markers are indicated in KD on the left.


1
2
3
4
5
6
200–
97–
69–

3.7.2. Characterisation of the EHV1 gene 71 protein.

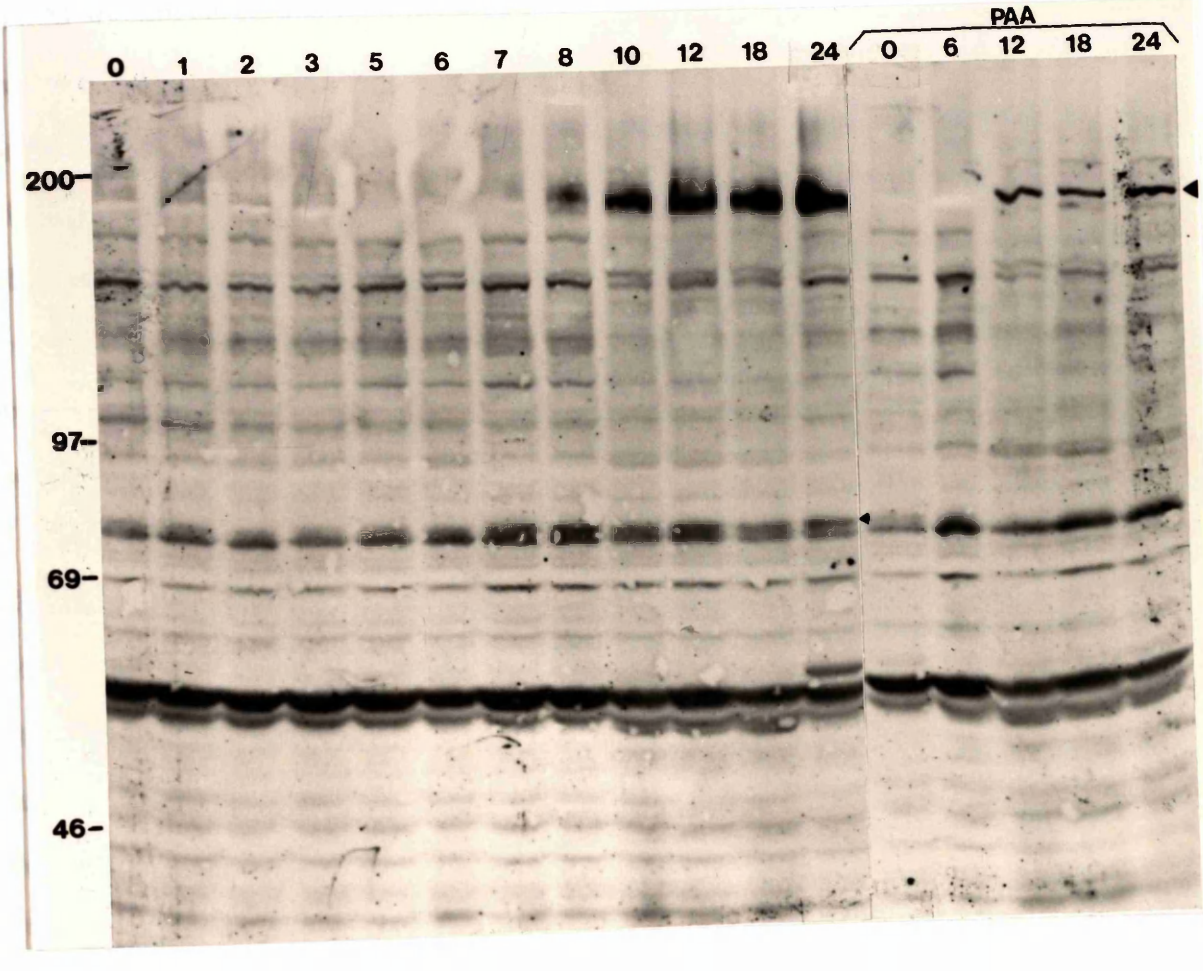
In this section the gene 71 protein was characterised in terms of regulation of expression, localization and post-translational modification.

3.7.2.1. Regulation of expression of the gene 71 protein

To study viral gene regulation, a time course of gene 71 expression was analysed. Monolayers of BHK21/C13 cells in 33mm diameter dishes were infected with virus at m.o.i. of 5 pfu/cell and harvested in sample buffer at various times after infection. The polypeptides in extracts were separated by 8% SDS-PAGE and transferred onto a nitrocellulose membrane and Western blotted using anti-71 serum. The results presented in Fig. 37 show that the 192 KD polypeptide of the gene 71 product was first detected at 8 h p.i.; the amount of expressed polypeptide increased until about 12 h p.i. and remained constant up to 24 h p.i. This pattern was reproducible in duplicate experiments and suggested that gene 71 is regulated as a late gene. To further determine the regulation of gene 71, expression of the 192 KD protein was examined in the presence of 300 μg/ml of PAA. As mentioned previously at this concentration of PAA, virus DNA replication is reduced to undetectable levels (<5% of the untreated control) in cells infected with both EHV1 and HSV1(Sun *et al.,* 1995; Johnson *et al.*, 1986). Infected cells were harvested at 0, 6, 12, 18 and 24 post-adsorption. The 192 KD Mr protein was first detected at 12 h after adsorption. Although virus DNA replication was not essential for gene 71 expression, it was significantly reduced in the presence of PAA (Fig.37, PAA). For comparison, regulation of expression of a HSV1 true late gene US11 (Johnson *et al.,* 1986) and a delayed early gene UL42 (Goodrich *et al.*, 1989) were also tested under the same conditions. The results showed that in the presence of PAA synthesis of the US11 encoded protein could not be detected at any time after infection, whereas the UL42 protein was clearly detected by 6 h p.i. and only slightly reduced by inhibition of PAA (data not shown). Overall results from these experiments demonstrated that gene 71 is regulated as a leaky late gene in EHV1 infected cells.

Figure 37. Time course of accumulation of the gene 71 product during infection

BHK21/C13 cells infected with EHV-1 strain Ab4 in the absence or the presence of 300μg/ml PAA were harvested at the times shown (in hours) after infection and the proteins extracted from the cells separated by 8% SDS-PAGE. Polypeptides were transferred to a nitrocellulose membrane and probed by anti-71 serum. The product of gene 71 is indicated by an arrow. Molecular weight markers are shown on the left in KD. PAA indicates the lysates of infected cells in the presence of PAA.


PAA
0 1 2 3 5 6 7 8 10 12 18 24
0 6 12 18 24
200
97
69
46

3.7.2.2. Location of the gene 71-encoded polypeptide within infected cells

The gene 71 protein was predicted to be a class I membrane protein. To determine whether the protein is associated with cellular membranes, monolayers of BHK21/C13 cells in 50 mm diameter dishes were infected with virus at a m.o.i. of 5 pfu/cell and at 16 h p.i., the cells were fractionated into a soluble cytosol fraction and a membrane-containing fraction by the method described in 2.2.24. The polypeptides in the fractions were separated by 8% SDS-PAGE and Western blotted with anti-71 serum. The results presented in Fig. 38 showed that the 192KD polypeptide is detected in the membrane fraction in the presence of 1 M NaCl and 100 mM-sodium carbonate, pH 11.5. (Fig. 38, lane 3). The 192 KD protein is not present either in the peripheral membrane fraction, the cytosol or in the supernatant (Fig. 38, lanes 4, 5 and 6). This result showed that the gene 71 protein has a high affinity association with cellular membranes confirming that the gene 71 protein is a membrane bound protein. In addition, a 80 KD species corresponding in size to the predicted primary translation product of the gene 71 protein was specifically detected in the cytosol fraction (lane 5) and in the total infected cell extract (lane 1), but was not present in mock infected cells nor in the membrane fraction (Fig. 38, lanes 2 and 3).

3.7.2.3. Virion localisation of the gene 71 polypeptide.

EHV1 virus preparations consist of virions and light particles (L particles). L particles are essentially envelope-tegument structures (McLauchlan & Rixon, 1992). To determine the location of the gene 71 protein within virions, purified virions and L particles were prepared and extracted with sample buffer. The extracted polypeptides were separated by 8% SDS-PAGE and Western blotted with anti-71 serum (Fig. 39a, lanes 1 and 2). For comparison of the amount of loaded protein, the same amounts of extracts from virions and L particles were run in adjacent lanes in the same gel and stained with Coomassie blue (Fig. 39 a, lanes 3 and 4). The gene 71 polypeptide was detected in both purified L particles and virions as a wide band with a molecular weight over 200 KD (Fig. 39, a, lanes 1 and 2). This result showed that the gene 71 protein was a virion component, present in the envelope and tegument.

Figure 38. Western blotting analysis of subcellular fractions with anti-71 serum.

Cells infected with EHV-1 Ab4 were fractionated as described in section 2.2.24. The fractionated polypeptides were then separated by 8% SDS-PAGE and Western blotted using anti-71 serum. EHV-1 infected cell extracts lanes 1, 3, 4, 5 and 6; Lane 1, total infected cell extract; Lane 2, mock infected cell extracts; Lane 3, the insoluble pellet of the membrane fraction treated with 1M NaCl and a pH 11.5 acid buffer; Lane 4, the soluble fraction of the treated membrane fraction; Lane 5, cytosol; Lane 6, culture supernatant. Molecular weight markers are shown on the left in KD. The products of gene 71 are indicated by arrows .

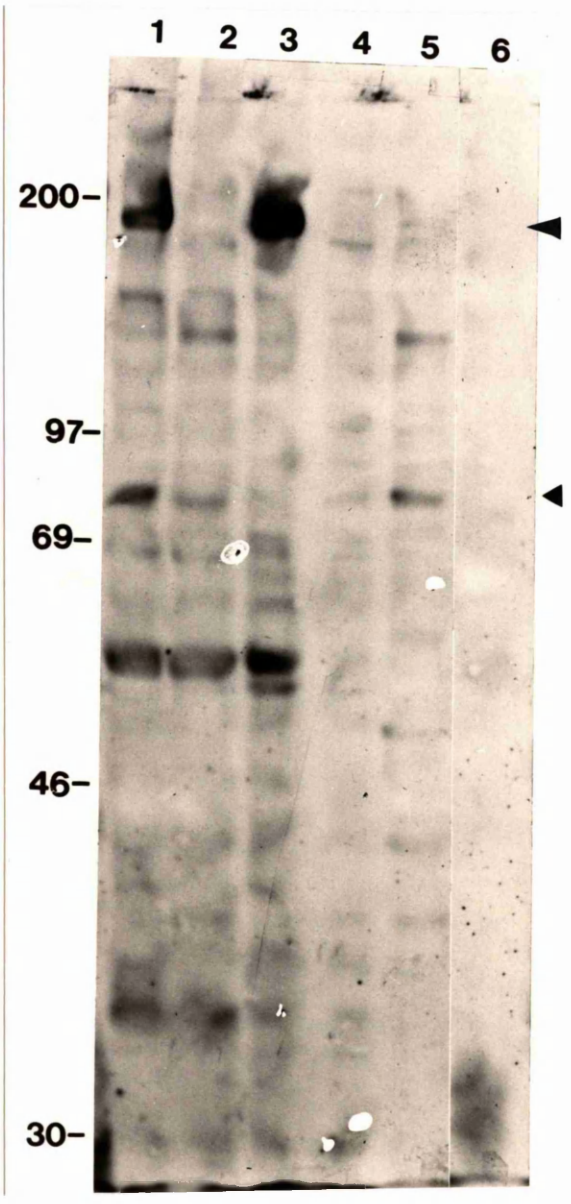
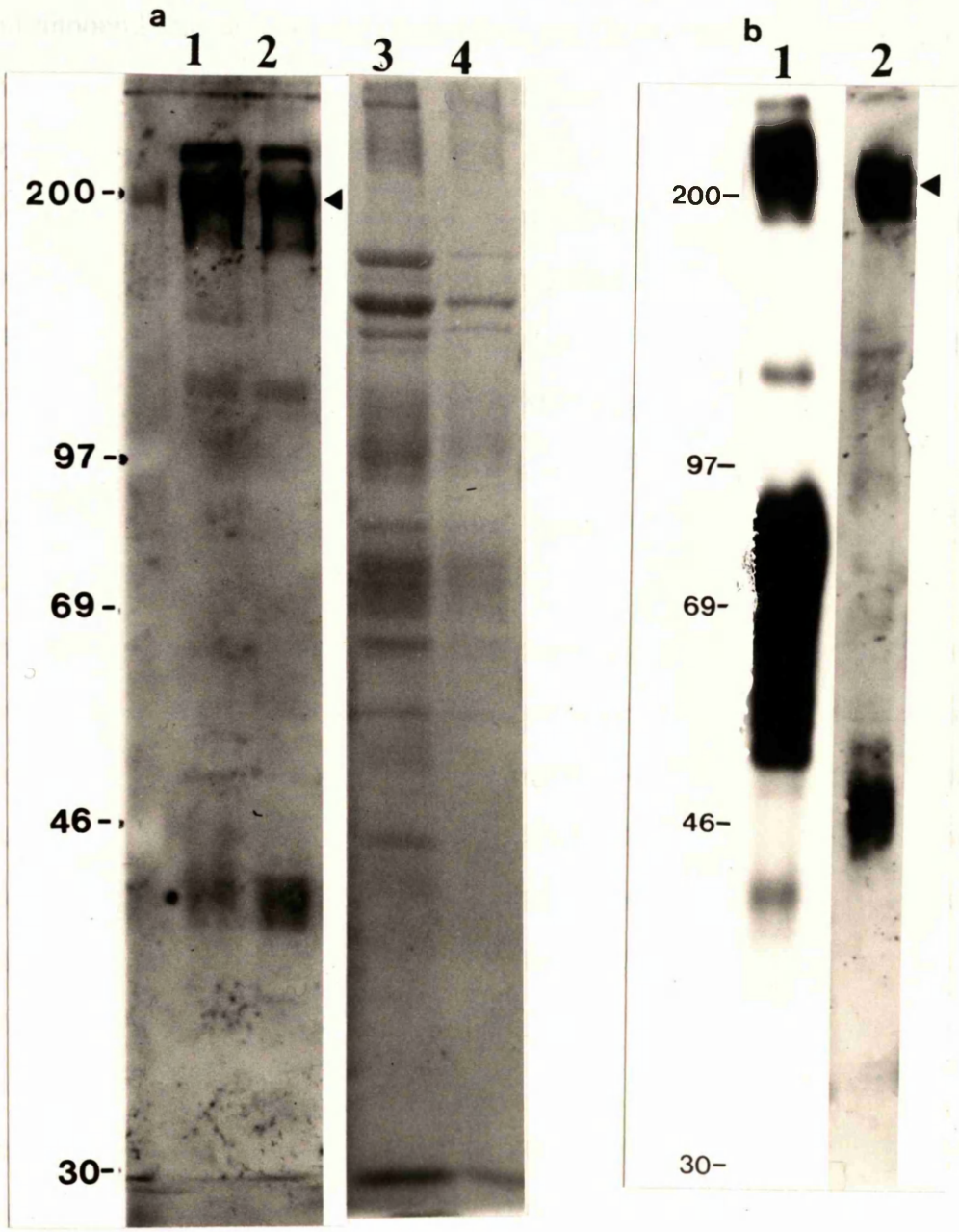

1
2
3
4
5
6
200–
97–
69–
46–
30–

Figure 39. (a) Western blotting analysis of purified virions and L-particles with anti-71.

Virions (lanes 1 and 3) and L particles (lanes 2 and 4), purified as described in section 2.2.11, were analysed by 8% SDS-PAGE. Lanes 1 and 2, virions and L particles probed with anti-71. Lanes 3 and 4, the same samples were loaded onto adjacent lanes and stained with Coomassie blue. The product of gene 71 is indicated by an arrow. Molecular weight markers are shown on the left in KD.

(b) Autoradiography of surface labelled virion proteins.

Purified virions were either labelled and analysed by 8% SDS-PAGE or analysed by western blotting with anti-71. Lane1, purified virions labelled with UDP-[^{14}C] galactose by the galactosyl transferase as described in methods. Lane 2, purified virions probed with anti-71. The gene 71 product is indicated by an arrow. Molecular weight markers are shown on the left in KD.


a
1
2
3
4
200
97
69
46
30
b
1
2
200
97
69
46
30

To further determine the precise location of the gene 71 protein, freshly purified virions were labelled *in vitro* with UDP-[^{14}C] galactose by enzymatic labelling which specifically labels the exposed carbohydrate residues on the virion envelope surface. Labelled virions were purified from unbound radioactivity and analysed by 8% SDS-PAGE, the gel dried and exposed to film. The size of the labelled gene 71 protein was determined by alignment with the gene 71 polypeptide recognised by immunoblotting with the anti-71 serum in the adjacent lane. The result shows that a high molecular weight virion polypeptide with the same molecular weight as that of the gene 71 polypeptide recognised by anti-71 (Fig. 39 b lane 2) was surface labelled by UDP-^{14}C galactose (Fig. 39 b, lane 1). This result suggests that the gene 71 polypeptide was present on the surface of the EHV1 virion envelope.

3.7.2.4. Post-translational modification of the gene 71-encoded polypeptide.

Sequence analysis showed that the gene 71 protein contains a single putative N-linked glycosylation site and an extensive region encoding a high proportion of serine and threonine residues (amino acids 22-465), which is likely to be heavily O-glycosylated (Telford *et al.*, 1992). It has been demonstrated that the gene 71 encoded polypeptide had a much higher molecular weight (192 KD) than the predicted primary product (80 KD). These data suggested that the gene 71 protein is likely to be a heavily O-linked glycoprotein leading to a high molecular weight on SDS-PAGE.

To determine whether the gene 71 protein is glycosylated, three experiments were carried out. Firstly, infected cells were labelled with [^{35}S] methionine, [^{3}H] mannose or [^{14}C] glucosamine from 3 h to 16 h p.i. ^{14}C glucosamine specifically labels the inner complex-O-linked carbohydrate chains on a glycoprotein. At the end of labelling, the cells were lysed with extraction buffer and the polypeptides immunoprecipitated with anti-71 and analysed by SDS-PAGE. The results showed that the gene 71 polypeptide was labelled with [^{35}S] methionine (Fig. 40a, lane 2) and [^{14}C] glucosamine (Fig. 40b, lane 2), but not by [^{3}H] mannose (data not shown). The polypeptide was not precipitated from mock infected cell

extracts by anti-71 (Fig. 40a,b lane 1) nor by preimmune serum from EHV1 infected cell extracts (Fig. 40a,b lane 3). A high M_r protein band (›200 KD) labelled with ^{14}C-glucosamine was also specifically precipitated by anti-71 (Fig. 40b, lane 2). Secondly, two metabolic inhibitors of glycosylation, tunicamycin and monensin, were used. Tunicamycin specifically inhibits transfer of N-acetyl glucosamine-1 phosphate from UDP-N-acetylglucosamine to dolichol monophosphate and thereby blocks the formation of protein-carbohydrate linkages of the N-glycosidic type (Mahoney and Duksin *et al.*, 1979). Monensin catalyses the exchange of sodium and potassium ions across biological membranes and is thought to disrupt ion gradients that appear to be crucial to the budding of vesicles from the Golgi complex (Pressman, 1976), thus inhibiting the processing of herpes simplex virus glycoproteins, particularly O-linked glycoproteins, in Golgi apparatus (Johnson, and Spear, 1982). Monolayers of BHK21/C13 were infected with virus at a m.o.i of 5 pfu/cell in the presence of tunicamycin (1µg/ml) or monensin (1 µM). The cells were harvested in sample buffer at 16 h p.i. and analysed by Western blotting with anti-71. The results showed that the apparent M_r of the gene 71 protein was reduced by 9KD following treatment with tunicamycin indicating that the gene 71 protein is N-glycosylated (Fig. 41 a, lane T). Treatment with monensin caused the disappearance of the high M_r form (192 KD) of the gene 71 protein and an increase in the amount of the 80 KD M_r polypeptide band (Fig. 41a, lane M), with the same M_r as that of the predicted primary gene 71 product. Thirdly, purified EHV1 virions were digested with glycanases. These enzymes include N-glycanase, which appears to hydrolyse the high mannose and complex forms present in the core region of N-linked oligosaccharides (Elder and Alexander, 1982); neuraminidase, which removes sialic acid and exoglycanidases including α-L-fucoside fucohydrolase, ß-galactosidase and ß-N-acetyl hexosaminidase. These exoglycanidases have been reported to liberate terminal ∂-L-fucoside, ß-linked galactose and ß-linked N-acetylglucosamine and N-acetylgalactosamine from a variety of substrates (Carlsen and Pierce, 1972; Bahl and Agarwal, 1969; Li and Li, 1970). Digested polypeptides were separated by 8% SDS PAGE and analysed by Western blotting with anti-71. The results showed that the apparent M_r of the gene 71 polypeptide was only slightly reduced by treatment with N-glycanase (Fig. 41b, lane N-g). Digestion with

Figure. 40. Autoradiography of precipitated gene 71 polypeptide with anti-71 serum

Cells labelled with (a) [^{35}S] methionine or (b) [^{14}C] glucosamine were extracted with lysis buffer and immunoprecipitated with either anti-71 (Lanes 1 and 2) or preimmune serum (Lane 3) and analysed by 8% SDS-PAGE. Lane 1, mock infection; Lanes 2 and 3, EHV1 infected cell extracts. The gene 71 product is indicated by a small arrow. A high M_r band is indicated by a large arrow. Molecular weight markers are shown on the left in KD.

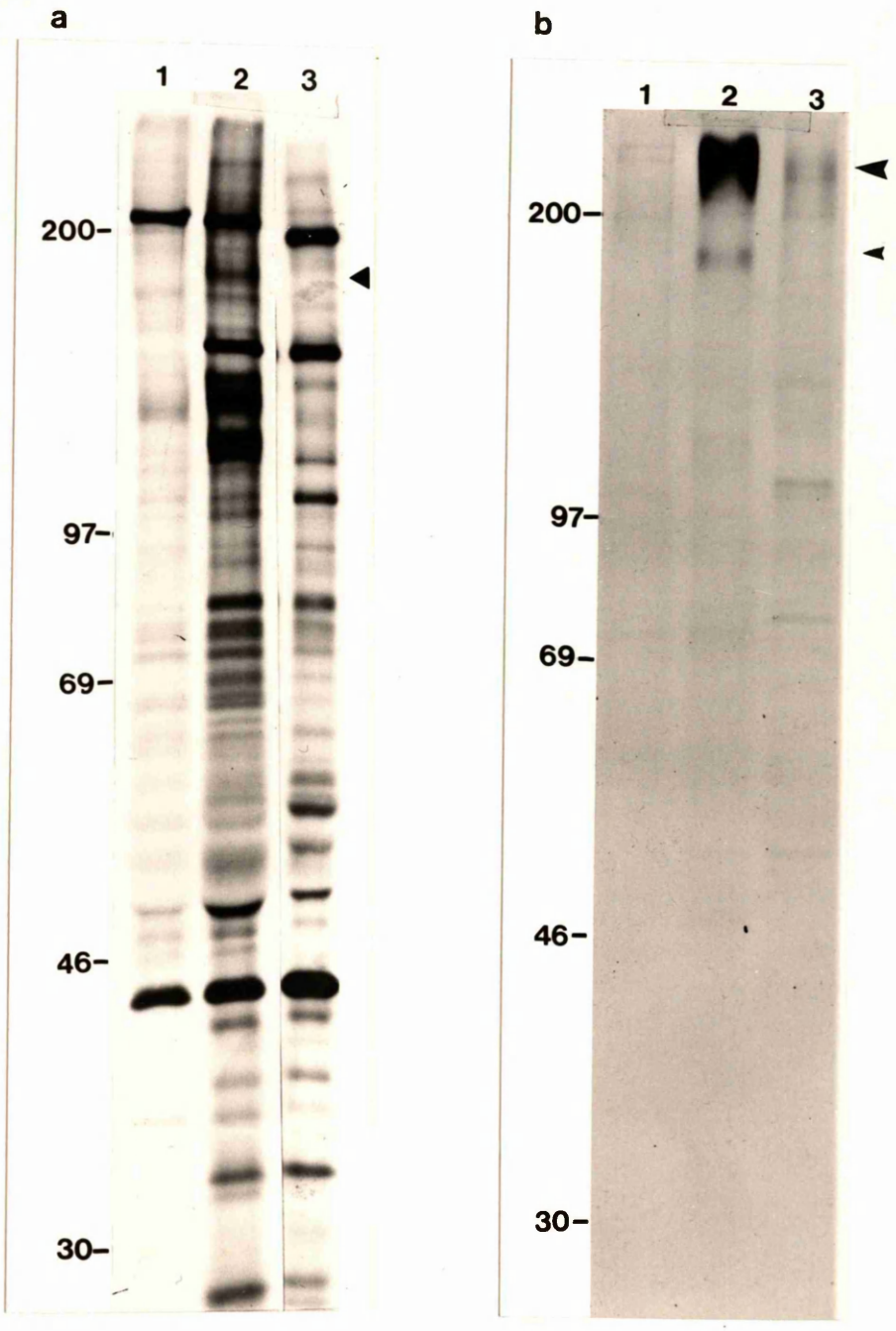

a
1
2
3
200-
97-
69-
46-
30-
b
1
2
3
200-
97-
69-
46-
30-

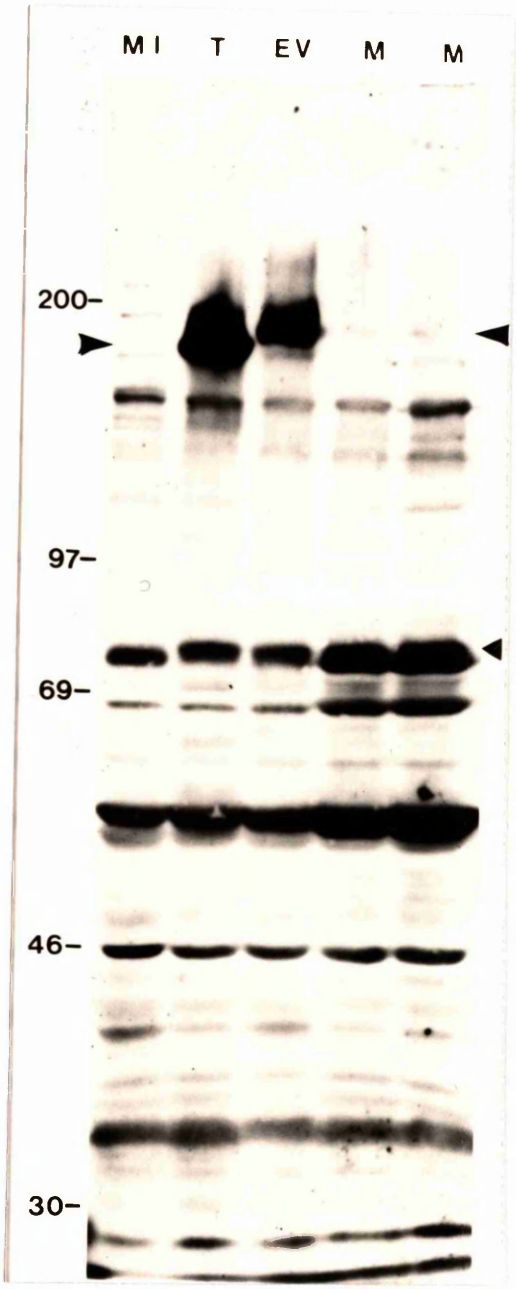
Figure 41. (a) western blotting of tunicamycin or monensin treated infected cell extracts

BHK21 cells were infected with EHV1 either in the presence or absence of tunicamycin or monensin. The cells were extracted at 16h p.i. Extracts from cells mock-infected (MI), infected with EHV-1 Ab4 (EV), infected with EHV-1 in the presence of tunicamycin (T) or monensin (M) were separated by 8% SDS-PAGE and probed by anti-71. The high molecular weight gene 71 polypeptide are indicated by large arrows and the 80 KD species by a small arrow. Molecular weight markers are indicated on the left in KD.

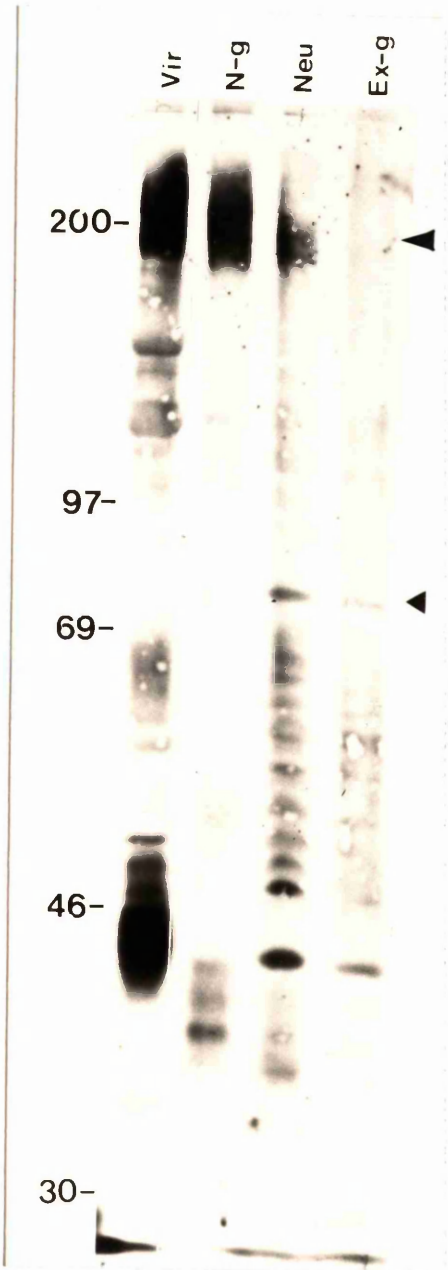
(b) Western blotting of glycanase digested EHV1 virions probed with anti-71

Purified EHV1 virions (Vir) were either directly extracted or digested with N-glycanase (N-g), neuraminidase (Neu), or exoglycanases (Ex-g) (ß-N-acetylhexosaminidase, L-fucosidase fusohydrolase and ß-galactosidase), prior to extraction with the sample buffer. The extracts were separated by 8% SDS-PAGE and Western blotted with anti-71. The gene 71 polypeptide is marked by a large arrow and the new species by a small arrow. Molecular weight values are shown on the left in KD.

a



b



neuraminidase reduced the molecular weight and amount of the high M_r form of the gene 71 protein and produced a new species with a 80 KD M_r (Fig. 41b, lane Neu). Finally digestion with exoglycanases completely removed the high M_r form of the gene 71 protein and also produced a band with 80 KD Mr (Fig. 41b, lane Ex-g), which corresponds to the 80 KD primary product of the gene 71 protein seen in infected cells. As a control, HSV1 strain 17^+ virions were digested under the same conditions with these glycanases and the digestion analysed by Western blotting with the antibody against HSV1 glycoprotein D (gD). The results showed that as expected the enzymes reduced the molecular weight of HSV1 gD (data not shown). Overall the results demonstrated that the high molecular weight gene 71-encoded polypeptide was heavily O-glycosylated and also contains limited N-linked oligosaccharides.

3.7.3. The relationship between the gene 71 protein and EHV1 glycoprotein gp300

The glycoprotein gp300 had been previously identified as an EHV1 membrane protein modified as a heavy O-linked glycoprotein with a high M_r over 400 KD on DATD cross linked SDS-PAGE (Allen & Yeargan, 1987; Whittaker *et al.*, 1990). It was also shown to be encoded by EHV1 gene 28 (Whittaker *et al.*, 1992a). During the present study it became apparent that the high M_r glycoprotein encoded by gene 71 of EHV1 Ab4 had very similar characteristics to gp300. Sequence analysis data suggested that the EHV1 gene 28 protein was not a typical class I membrane protein and not likely to be a heavily O-linked glycoprotein (Sun *et al.*, 1994). The accumulated data therefore raise the question whether the two EHV1 proteins are the same polypeptide and if so, which gene encodes the protein.

To determine if gp300 recognised by P19 and the gene 71 protein recognised by anti-71 were the same protein, the following experiment was carried out. Polypeptides from EHV1, ED71 and the revertant, Re-71 infected cells were extracted and analysed by Western blotting with anti-71 serum which is specific for the gene 71 protein and a monoclonal antibody P19 which specifically recognises gp300 (Whittaker *et al.*, 1990) on the same gels. The results showed that a polypeptide identical to gp300 was recognised by P19 and the anti-71 serum from

EHV1 and Re71 infected cell extracts on 8% SDS-PAGE cross-linked with either bis-acrylamide (a) or N',N'-diallyltratardiamide (DATD) (b) (Fig. 42, lanes 1, 2, 6, 7, 8, 12). Both antibodies, P19 and anti-71, detected the same M_r shift following treatment with tunicamycin (Fig. 42, lanes 5, and 11). Neither P19 nor anti-71 detected gp300 or the gene 71 protein in mock infected or ED71 infected cell extracts (Fig. 42, lanes 3, 4, 9 and 10), although the anti-71 serum recognised the 116k M_r ß-galactosidase polypeptide (lane 10).

Overall these results demonstrated that the protein (gp300) recognised by MAb P19 is the same polypeptide as the product of gene 71 identified by anti-71 and that this polypeptide is encoded by EHV1 gene 71 and not by EHV1 gene 28 as previously published (Whittaker *et al.,* 1992a).

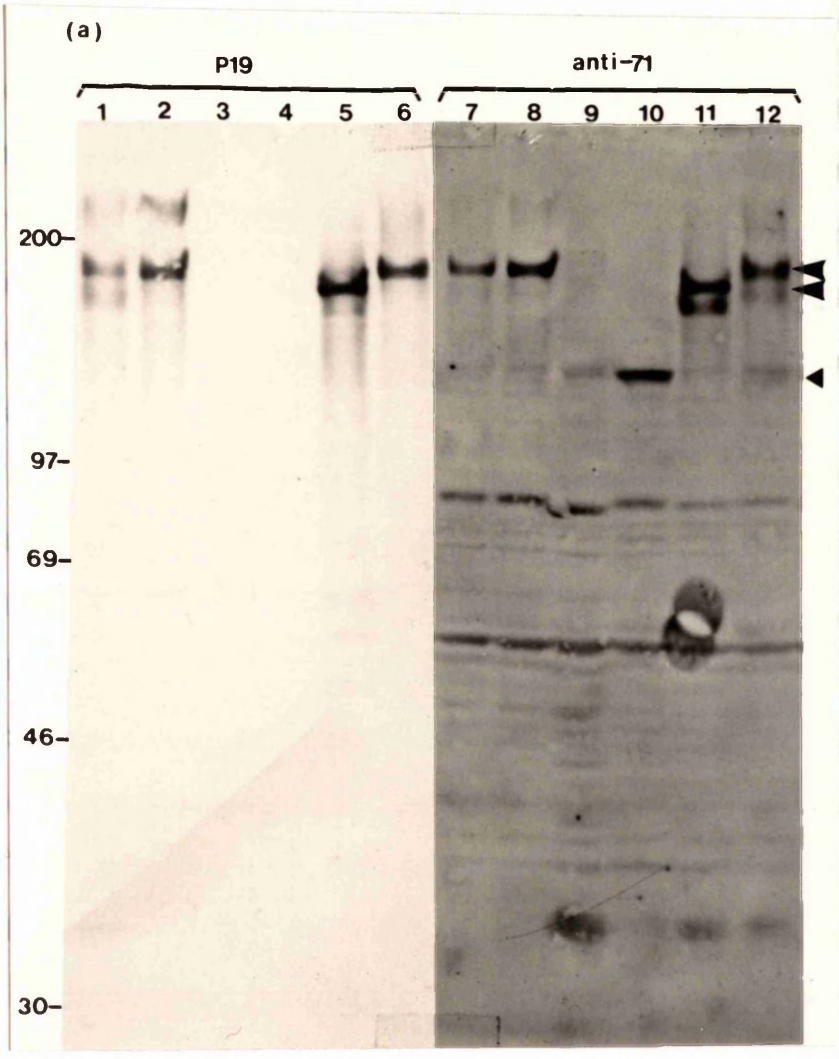
A high M_r infected cell polypeptide (>200 KD) was also picked up by both antibodies (P19 and anti-71) along with the gp300 and the gene 71 protein, this protein was more strongly recognised by p19 than by anti-71 (Fig. 42 lanes 1, 2, 6, 7, 8 and 12). This high M_r polypeptide was not present in ED71 infected cell extracts and its M_r was reduced following treatment with tunicamycin (Fig.42 lanes 5 and 11). A similar M_r protein heavily labelled with ^{14}C glucosamine was also precipitated by anti-71 (Fig. 40b, lane 2). These data indicate that this high M_r protein is likely to be a highly glycosylated form of the gene 71 protein, which appears to be the predominant form recognised by P19.

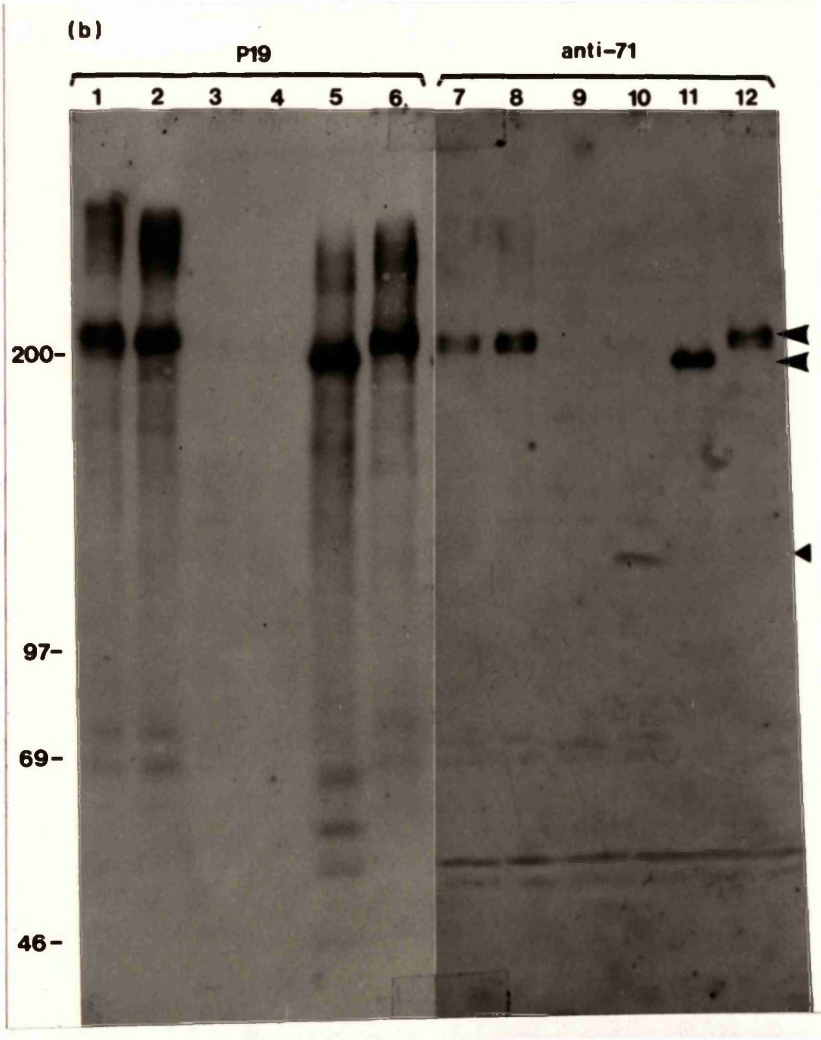
3.8. CHARACTERISATION OF THE DELETION MUTANT OF EQUINE HERPESVIRUS TYPE 1 GENE 71, ED71

The results from section 3.4. showed that deletion of EHV1 gene 71 obviously impaired the growth properties of the virus *in vitro*, the final titre of ED71 being 5- to 10-fold lower than that of wild-type virus or its revertant. ED71 was also found to be attenuated in its pathogenicity in a murine model (Tim Fitzmaurice, personal conmunication). These features of ED71 suggested that the gene 71 protein plays an important role in the virus life cycle which could not be fully complemented by host proteins. Characterization of ED71 could

Figure. 42. Western blotting of the EHV1 gene 71 product and gp300 probed with anti-71 serum and P19 monoclonal antibody.

Extracts from cells mock-infected (lanes 3 and 9), infected with mutant ED71 (lanes 4 and 10), infected with revertant Re71 (lanes 1 and 7), infected with EHV-1 Ab4 (lanes 2, 6, 8 and 12) and infected with EHV-1 Ab4 in the presence of tunicamycin (1µg/ml) (lanes 5 and 11) were analysed by 8% SDS-PAGE cross-linked with bisacrylamide (a), or DATD (b) and probed with anti-71 serum (lanes 7-12) and the P19 monoclonal antibody (lanes 1-6). The gene 71 polypeptide and gp300 are indicated by large arrows and the 116K ß-galactosidase band is indicated by a small arrow. Molecular weight values are shown on the left in KD.


(a)
P19
anti-71
1 2 3 4 5 6 7 8 9 10 11 12
200-
97-
69-
46-
30-


(b)
P19
anti-71
1 2 3 4 5 6 7 8 9 10 11 12
200-
97-
69-
46-

reveal the role of the gene 71 protein in the virus life cycle and provide data to explain the reason for the attenuation of ED71 *in vivo*. Since the gene 71 protein was demonstrated to be a class I membrane glycoprotein, it possibly plays a role in virus entry into cells, egress from cells and transmission from cell to cell. The deletion mutant (ED71) was characterised in terms of entry into cells, egress from cells and transmission from cell to cell, compared to the wild type virus and its revertant, Re71.

3.8.1 ED71 entry into cells

To determine the efficiency of ED71 adsorption onto cells, precooled monolayers of BHK21/C13 cells were infected with 300 pfu/plate of purified EHV1 virions and adsorption allowed to proceed for various times at 4°C. At each time point the plates were washed thoroughly with PBS and incubated at 37°C. After two days, the plates were stained and the plaques counted. Virus adsorption is presented as a percentage of pfu from the final time point plates (4 hr). Each point on the graph represented an average of 3 plates. The results represented in Fig 43. show that purified ED71 adsorbs more slowly onto cells than wild-type and revertant virus at 4°C. Its 50% adsorption time being 110 min compared to 60 min for wild type and revertant. These experiments were repeated several times with similar results. A similar effect was also observed in an experiment performed at 37°C (data not shown). These results demonstrated that the rate of adsorption of ED71 virions onto cells is obviously lower than that of wild-type virus and Re71.

To determine whether there is a defect in penetration during entry of ED71 into cells, monolayers of BHK21/C13 cells in 50 mm plates were infected with 400 pfu/plate of purified virions which were allowed to adsorb for 2h at 4°C. After washing with PBS, the infected cells were shifted to 37°C and treated with either an acid buffer (pH3.0) or PBS (as a control) for 5 min at various times after shift. This protocol is described in 2.2.14. The numbers of plaques on the plates, which was treated with acid buffer, was determined and compared with that of the untreated control. Acid resistance represents the efficiency of virion penetration and is taken as a measure of virion penetration. The results (Fig. 44) show that penetration of

Figure. 43. Adsorption of purified ED71, Re71 and wild-type virus onto BHK21/C13 cells.

Monolayers were infected with 300 pfu of virus and adsorption allowed to take place for various times at 4°C. Unbound virus was washed off with PBS and the cells overlaid with medium containing methylcellulose and incubated at 37°C. After 2 days the cells were stained and the plaques counted. The rate of virus adsorption was expressed as a percentage of the control (the numbers of plaques from the 4 h time-point plate). Results are averages from an experiment done in triplicate and error bars indicate standard deviations.

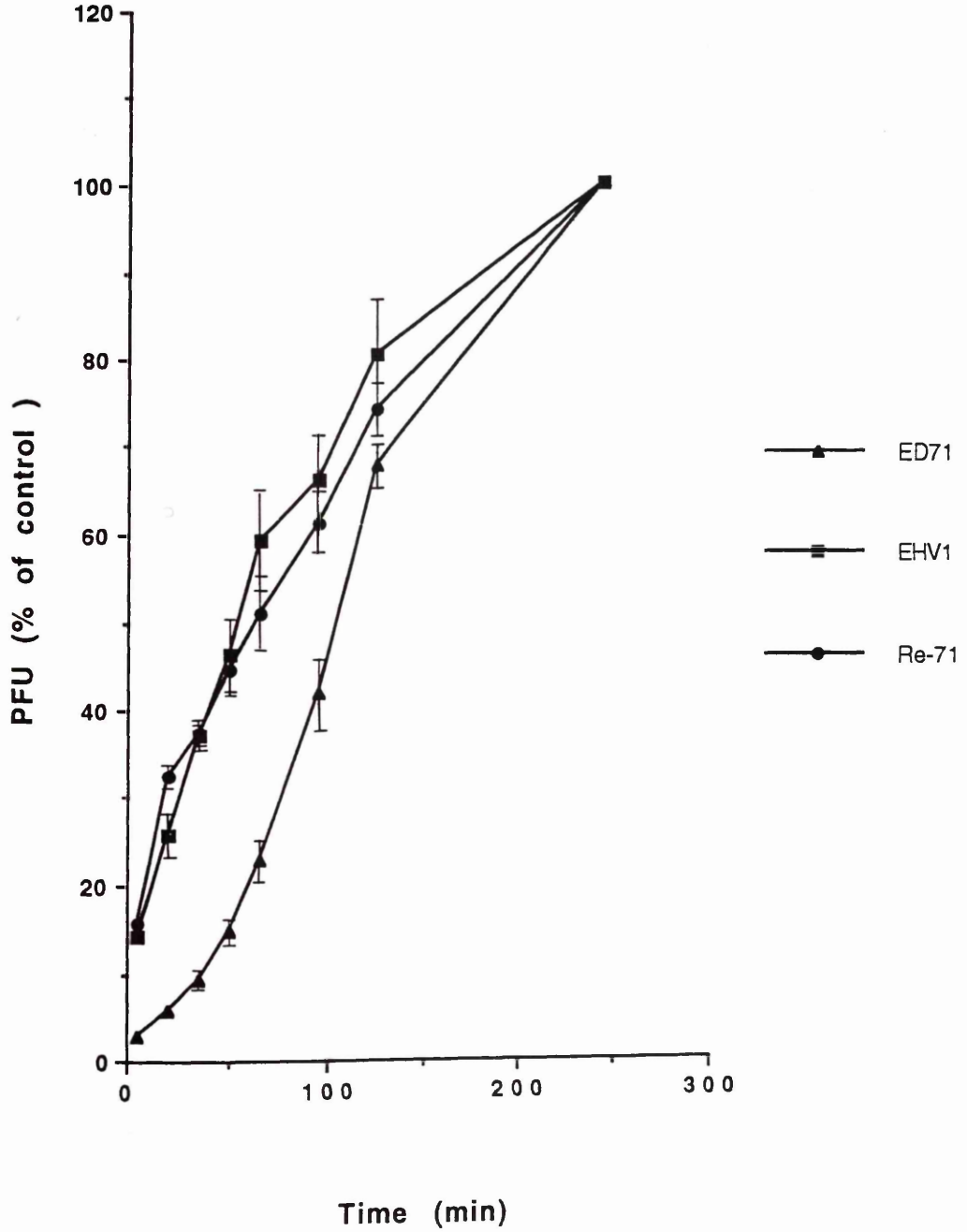
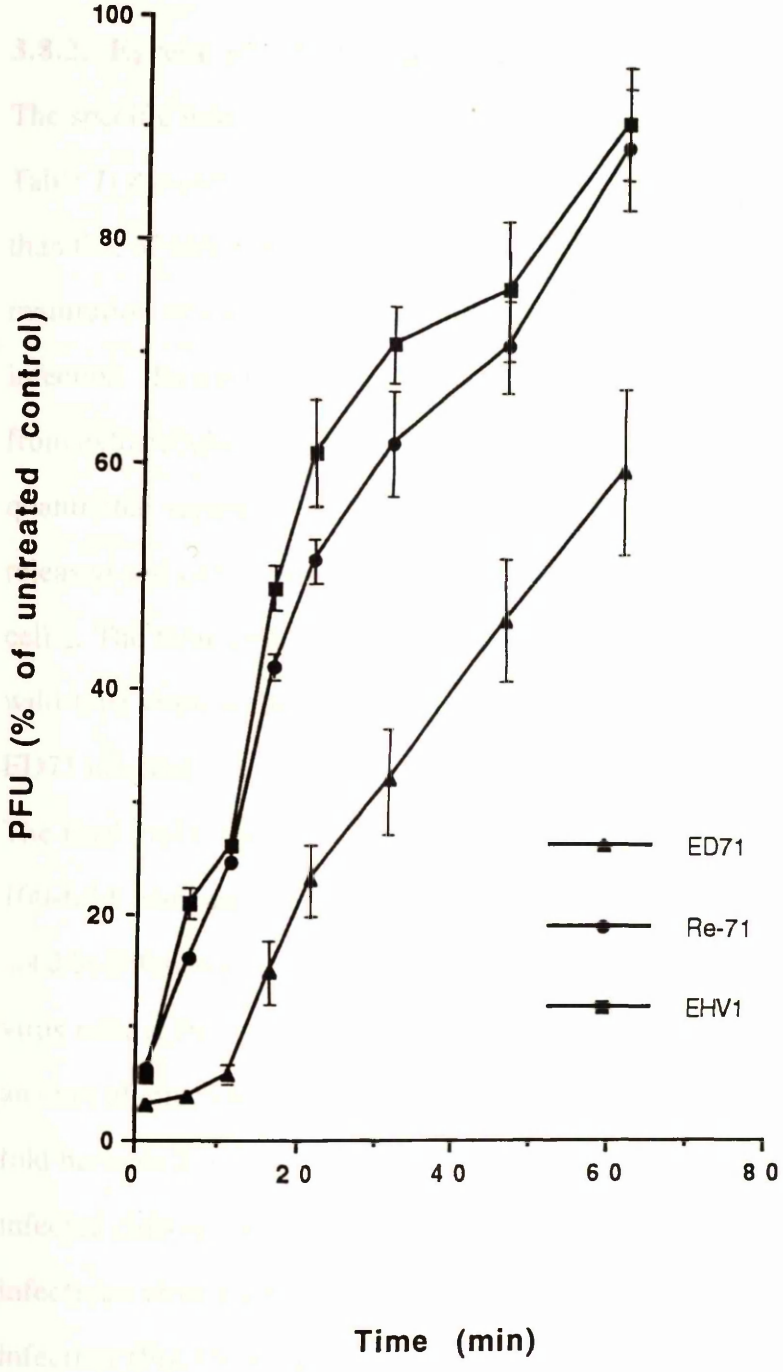

120
100
80
60
40
20
0
PFU (% of control)
0
100
200
300
Time (min)
ED71
EHV1
Re-71

Figure 44. Penetration by ED71, Re71 and wild-type virus into BHK21/ C13 cells.

Monolayers of cells were inoculated with 400 pfu of purified virions for 2 h at 4°C to allow adsorption to take place. The monolayers were then shifted to 37°C. At indicated time points, monolayers were treated for 5 min with 1 ml of citric acid buffer or with PBS. The monolayers were overlaid with medium containing methylcellulose and incubated at 37°C for 2 days and plaques counted. The virus surviving from citrate treatment at each time point is presented as a percentage of the PBS treated control value. Each time point value is the average of three plates and the error bars represent standard deviations.


100
80
60
40
20
0
PFU (% of untreated control)
0
20
40
60
80
Time (min)
ED71
Re-71
EHV1

ED71 as a percent of the untreated control was significantly lower than that of wild-type and revertant. For 50% penetration, ED71 virions took 55-60 min, while wild-type virus took only 20 min. This experiment was repeated several times with similar results. Re71 showed a very similar penetration rate to wild-type virus. These results demonstrated that the penetration of adsorbed ED71 into cells is less efficient than for wild-type virus and Re71.

3.8.2. Egress of ED71 from cells

The specific infectivity of the virions purified from infected BHK21/C13 cells (presented in Table 7) showed that the particle/pfu ratio of purified ED71 is significantly higher (10-20 fold) than that of wild-type virus and Re71, suggesting that there could be a defect in ED71 virion maturation or egress. Similar results were obtained following both high and low m.o.i. infection. To test if ED71 has a defect in release from infected cells, infectious progeny virus from extracellular and intracellular fractions at various times post infection was harvested and quantitated separately. The results presented in Fig. 45 compared the yields of total, cell released and cell associated infectious virus from ED71, wild-type virus and Re71 infected cells. The total yield from ED71 infected cells decreased by 7 fold compared with that of wild-type virus and revertant (Fig.45, Total). The yield of infectious progeny released from ED71 infected cells is significantly lower than that from wild-type and revertant infected cells. The final yield of released infectious virus from ED71 infected cells decreased about about 100-fold compared to wild-type and Re71 infected cells (Fig.45 cell-relased). Another notable difference between ED71 and wild-type virus infection was that the onset of infectious virus release from ED71 infected cells was significantly delayed (Fig 45 cell-released). The amount of infectious wild-type virus and Re71 released from infected cells increased by 1000-fold between 8 to 16 h post infection, whereas no significant release of infectious ED71 from infected cells occurred until after 16 h post infection (Fig. 45, cell-released). The yield of infectious virus from ED71 infected cells increased by only 20-fold from 16 to 24 h post infection (Fig.45 cell-released). The yields of cell-associated infectious virus from ED71 infected cell are about 2 to 3 fold lower than that from wild-type and revertant virus infected cells (Fig. 45 cell-associated). These observations show that egress of ED71 from infected

Table 7. The specific infectivity (particle/pfu) of ED71, Re71 and wild-type virus (EHV1).

Virus	*particle/pfu	"particle/pfu
EHV1	101.7/1	63.8/1
Re71	103.5/1	107.7/1
ED71	1440/1	2128/1

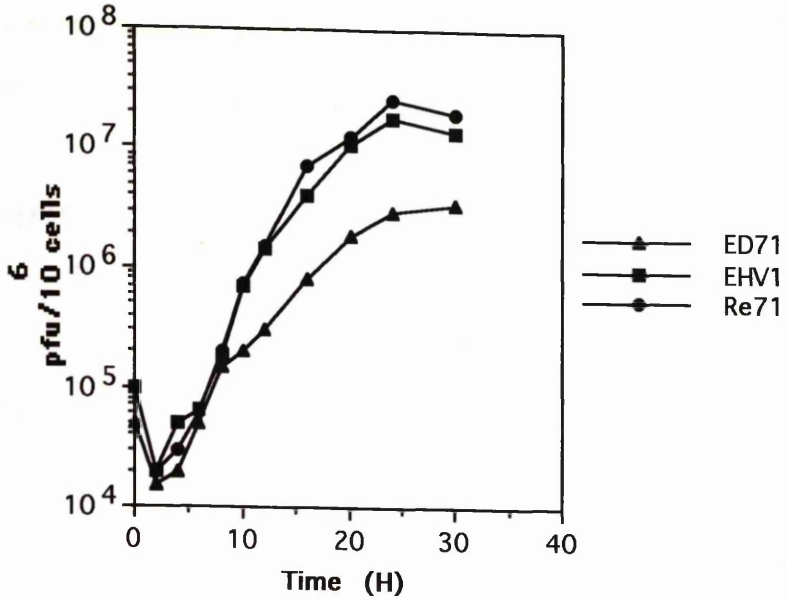
* Virus from $4x10^8$ BHK21/C13 cells infected at a m.o.i. of 0.01 pfu per cell and harvested at 72 h post infection.

"Purified virions from $4x10^8$ BHK21/C13 cells infected with virus at a m.o.i. of 5 pfu/cell and harvested at 20 h post infection.

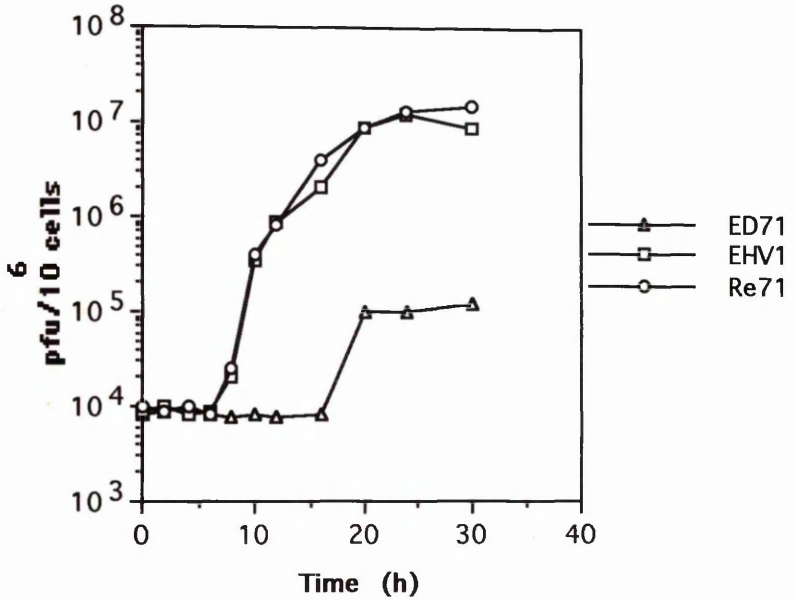
Figure. 45. Quantitation of cell-released infectious virus from the wild-type virus (EHV1), Re71 and ED71 infected cells.

BHK21/C13 monolayers were infected with virus at a m.o.i. of 5 pfu/cell and cell-released (CR) and cell-associated (CA) infectious virus harvested separately at various times (indicated in h) post infection were titrated in BHK21/C13 cells. Total graph shows total infectious virus titres. Cell-released graph shows cell-released infectious virus titres. Cell-associated graph shows cell-associated infectious virus titres. The titres at each time on the graph are shown as pfu/10^6 cells. ED71: infectious virus from ED71 infected cells; EHV1: infectious virus from wild-type virus infected cells; Re71: infectious virus from revertant infected cells.

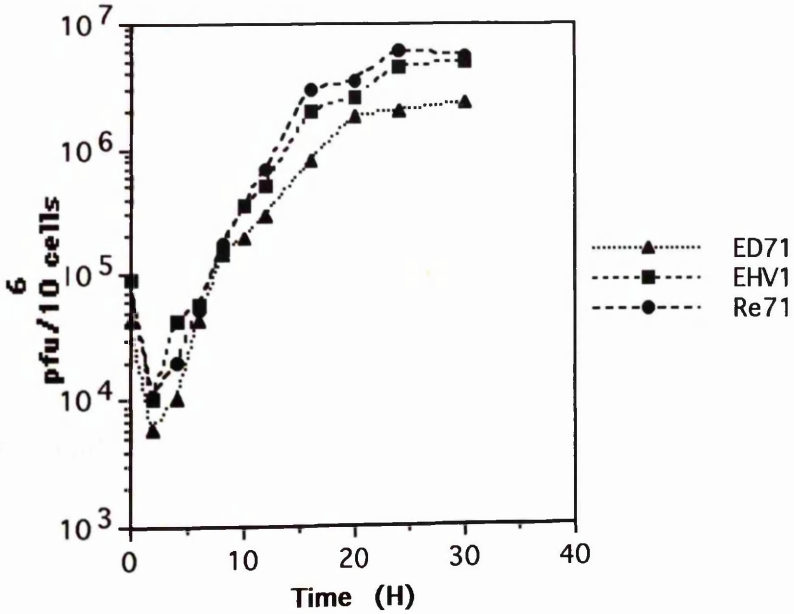
Total



Cell-released



Cell-associated



cells is impaired and that this defect is due to deletion of gene 71, suggesting that deletion of gene 71 greatly impairs virus release from cells to the extracellular space.

3.8.3. ED71 virion maturation

To examine ED71 virion maturation within cells, the distribution of capsids and virions in infected cells was analysed by electron microscopy. BHK21/C13 monolayers(4×10^6 cells) infected with virus at 3 pfu/cell, were scraped from petri dishes at 24 h post infection and pelleted in Been Capsules (Taab Laboratories). The pellet was fixed and 80 nm sections cut and stained. For counting, 50 complete cell profiles (non-serial and including nuclei) were examined (the cell fixing, sectioning and electron microscope examination were carried out by James D. Aitken). The results presented in Table 8 show that all three viruses produce very similar numbers (Total) of capsids in infected cells. However, in ED71 infected cells, a large number of capsids accumulate in the nuclei adjacent to the nuclear membranes, and the percentage of capsids in the ED 71 infected cell nuclei is much higher than that in wild-type and Re71 infected cells (92% compared to 73-77%). Another notable difference between ED71 and wild-type virus is the low number of particles (6.7% compared to 20% of total particles) and the low ratio of enveloped to unenveloped capsids (1% of ED71 cytoplasmic capsids were enveloped compared to 8% for w.t.) in the cytoplasm of ED71 infected cells. In addition, the percentage of extracellular virions from ED71 infected cells was much less than that from wild-type infected cells (0.8% compared to 2.4 %). These observations indicated that virion maturation is decreased in ED71 infected cells and could be an explanation for the higher particle/pfu ratio of purified ED71 virions and the defective growth of ED71. Since the revertant Re71 showed a similar pattern to wild-type virus deletion of gene 71 is responsible for this decrease.

3.8.4. Cell to cell transmission of ED71

Removal of the gene 71 product results in a defect in virus adsorption/penetration and virus egress from infected cells. This could imply that transmission of ED71 from cell to cell via

Table 8. Distribution of capsids in different subcellular locations of infected BHK21/C13 cells*

Location and condition	No. (%) of total capsids		
	EHV1, Ab4	ED71	Re71
Nucleus			
Total	1649 (77.6)	1443 (92.5)	1200(73.1)
Inside the nucleus	1649 (77.6)	1443 (92.5)	1200(73.1)
Between the lamellae	0	0	0
Cytoplasm			
Total	425 (20.0)	104 (6.7)	389 (23.7)
"Enveloped(%)	8	1	17
"Unenveloped(%)	92	99	83
Extracellular	51 (2.4)	13 (0.8)	52 (3.1)
Total (all locations)	2125 (100)	1560 (100)	1641 (100)

* The data is from 50 randomly selected sections of BHK21/C13 cells infected with virus at pfu/cell and harvested at 24 h post infection.

" Percent of total cytoplasmic capsids.

the extracellular medium could be affected and that virus transmission mainly occurs via direct cell to cell spread. To determine if this is the case, BHK21/C13 monolayers were infected with ED71, Re71 and wild-type virus at 100 pfu/plate. After 1 h adsorption, the cells were overlayed with EMC10 in the presence or absence of a horse antiserum (1:25), which neutralises the infectivity of ED71 and wild-type virus equally (data not shown), and incubated at 37°C for 2 days. The plates were stained and the plaques counted and photographed. The effect of neutralising antiserum on plaque size was examined. The results summarised in Table 9 and illustrated in Fig. 46 show ED71 formed smaller and tighter plaques than those of wild-type and revertant in the absence of antiserum. In the presence of antiserum, the plaques formed by wild-type virus and revertant virus were reduced and became similar to those formed by ED71. In contrast the plaques formed by ED71 were only marginally reduced by the presence of neutralising antiserum. This observation indicated that mutant release and readsorption transmission was impaired and the plaques formed by ED71 mainly arise from direct cell to cell transmission which consequently results in smaller and tighter plaques. The plaques formed by wild-type and revertant virus are the consequence of both direct cell to cell transmission and release and readsorption resulting in larger plaques. This data confirmed the conclusion that deletion of gene 71 impairs virus entry into cells and release from cells.

To further examine the transmission of ED71 *in vitro*, BHK21/C13 monolayers were infected with ED71 and wild-type virus at a low multiplicity of infection (0.01pfu/cell) and incubated in the presence or absence of the anti-EHV1 horse serum. At various times after adsorption, the cell-associated infectious virus (CA) was harvested and titrated on BHK21/C13 cells. The result in Fig. 47 showed that the yield of infectious progeny from ED71 infected cells was decreased only about 5-10 fold in the presence of the antiserum, whereas the yields of wild-type and Re71 progeny were reduced about 80-100 fold by the neutralising antiserum. In the presence of the antiserum, the yield of infectious cell-associated virus from ED71 infected cells was only 2 fold lower than that from wild-type and revertant, compared to 10-20 fold in its absence. This result indicated that spread of ED71 via release and readsorption is

Figure 46. Effect of antiserum on the sizes of plaques formed by the wild-type (W.T.), Re71 and ED71 on BHK21/C13 cells.

Wild-type virus (W.T.), ED71 revertant (Re71) and mutant (ED71) were assayed on BHK21/C13 cells and overlaid either with 1% carboxyl methylcellulose medium (-) or with 1% carboxyl methylcellulose medium containing neutralising horse antiserum at a dilution of 1:25 (+). After two days the cells were stained and plaques photographed.

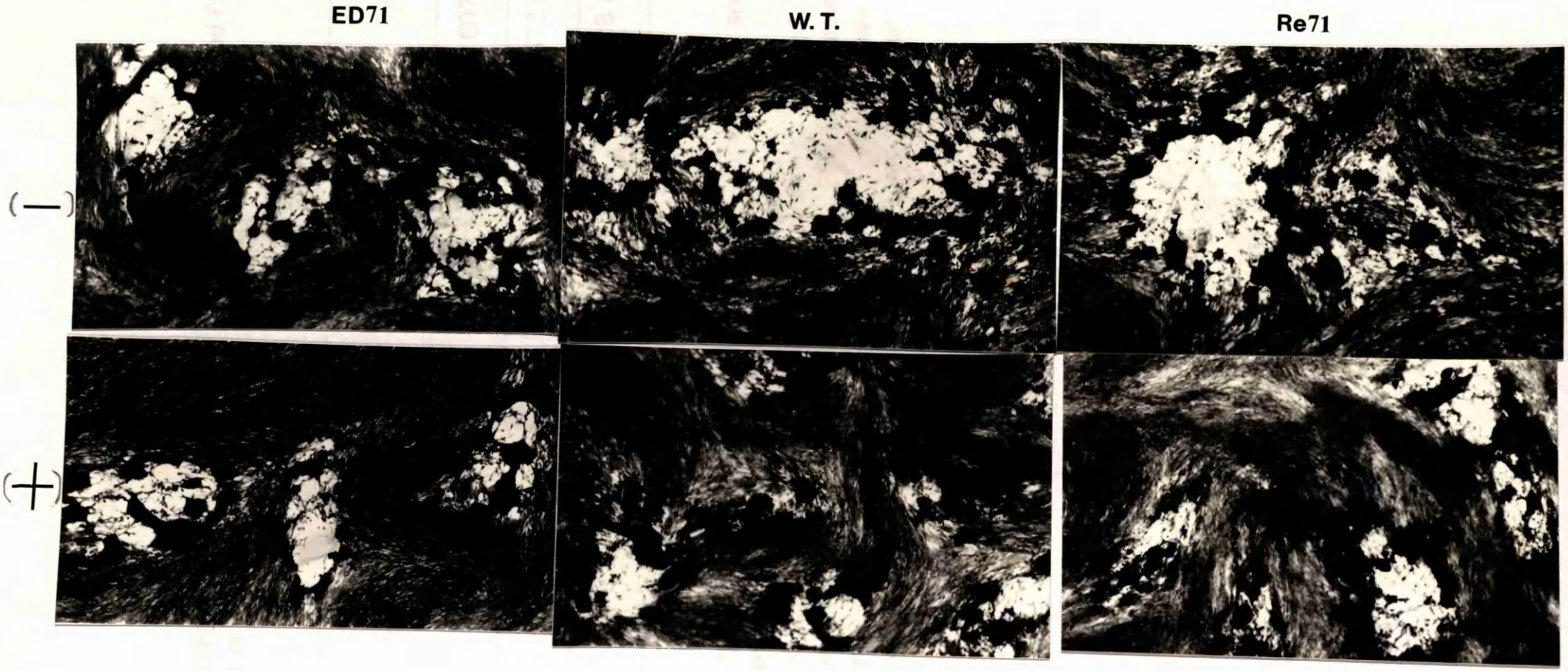

ED71
W. T.
Re71
(—)
(+)

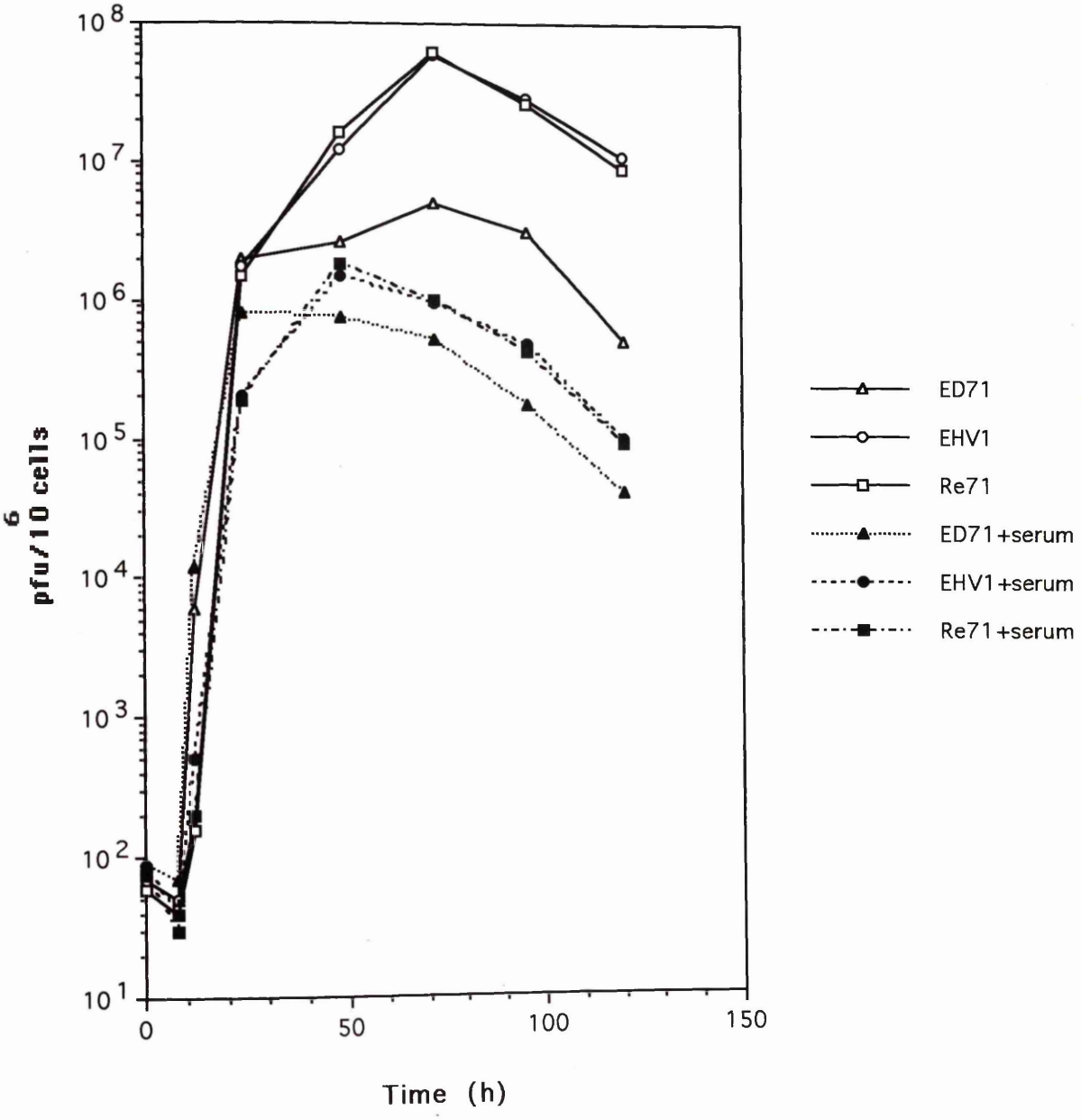
Table 9. The sizes of plaques* formed by wild-type, revertant virus and ED71 in the presence or absence of a horse neutralizing anti-EHV1 serum

Antiserum	Average plaque weight (gram)		
	EHV1	Re71	ED71
–	2.74 (±0.18)	2.61 (±0.3)	0.572 (±0.041)
+	0.492 (±0.065)	0.480 (±0.05)	0.428 (±0.031)

*Monolayers of BHK21/C13 cells were infected with the indicated virus and overlaid with methylcellulose(-) or with methylcellulose containing a neutralizing horse antiserum (1:25) (+). The cells were fixed and stained at 2 days post infection. 100 randonly chosen plaques were photographed in a OLYMPUS CR2 microscope and cut out from photographs. The plaque size of each virus is represented as average weight (gram) of the photographed plaques.

Figure 47. Effect of neutralising antiserum on the yield of ED71

Monolayers of BHK21/C13 cells were infected with 0.01pfu/cell of virus in the presence (+serum) or absence of a neutralising horse antiserum. Cell associated virus was harvested at the indicated time points p.i. The virus titres were determined on BHK21/C13 cells and are presented on the graph as pfu/10^6cells. Symbols: ED71: virus from ED71 infected cells; EHV1: virus from EHV1 infected cells; Re71: virus from Re71 infected cells.


10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
pfu/10 cells
6
0
50
100
150
Time (h)
ED71
EHV1
Re71
ED71+serum
EHV1+serum
Re71+serum

impaired, compared with that of wild-type and revertant, and the transmission of ED71 *in vitro* (BHK21/C13 cells) is mainly mediated by direct cell to cell spread.

Again as observed in Section 3.4 the cell associated titre of ED71 during the first 24h p.i. was higher than that of wild-type and Re71 (Fig. 47). Since the yield from Re71 infected cells are similar to that from wild-type virus infected cells, the increase is attributable to the deletion of gene 71. This is discussed in Section 4.7.

CHAPTER 4. DISCUSSION

4. 1. OBJECTIVES

The aim of the study presented in this thesis was to investigate the role of five EHV1 genes, 1, 2, 67, 71 and 75 in the virus life cycle. It included three objectives: (i) Generation and characterisation of individual deletion mutants in each of these genes to determine if they are essential and the effects on the virus life cycle. (ii) Characterisation of mutants with a detailed analysis of mutants in genes whose deletion impaired the growth of EHV1, and (iii) Identification and characterisation of the gene products of the five genes.

4. 2. GENERATION OF DELETION MUTANTS

Mammalian and avian herpesviruses in all the subfamiles possess a set of some 40 core genes (Davison, 1993) which have been inherited from a common ancestral virus. They represent all the major functional classes of genes, especially DNA replication, capsid structure and virion assembly, and their activities are seen as necessary for the function of a herpesvirus. The non core genes are representative of a particular subfamily, certain are specific to a member of a subfamily. Most genes involved in gene regulation, pathogenesis, latency, components of the envelope and tegument and in immune evasion are non core genes. Sequence analysis indicated that EHV-1 strain Ab4 genes 1, 67 and 75 have no homologues in the human herpesviruses, VZV and HSV1, and although genes 2 and 71 correspond in location to VZV gene 1 and HSV1 gene US 5, respectively, there is no similarity between their amino acid sequences (Telford *et al.*, 1992). This suggests that these genes are not conserved in herpesvirus subfamilies, appear to be non core genes, and these might play specific roles in the horse i.e. determination of pathogenesis.

In an attempt to investigate the roles of these genes in the EHV-1 replication cycle, an individual deletion mutant in each of the 5 target genes was generated. To delete the target genes, the majority of the target gene ORF was removed using restriction enzyme digestion. The deletions

were also made in such a way that the likelihood of disruption of adjacent genes was minimised. To aid in distinguishing mutant from wild-type virus, the deleted region was replaced by the *E. coli. lacZ* gene as a marker gene. In addition, an in frame stop codon was created between the remaining N-terminal portion of the target gene and the *lacZ* insertion to prevent the synthesis of a ß-galactosidase fusion protein.

Genes 1 and 2 are transcribed in a tail to tail pattern and their putative 3'-terminal polyadenylation (poly A) signals do not overlap (Fig. 2) (Telford *et al.,* 1992). To construct a deletion mutant in gene 1, 87% of the coding sequence was deleted. The deletion terminates 64 bp downstream from the stop codon of gene 2. To construct a deletion mutant in gene 2, 73% of the coding sequence was removed. The deletion extends from 92 bp downstream of the stop codon of gene 1, to 443 bp upstream of the start codon of gene 3. Gene 66 lies leftward and in a tail to tail orientation with gene 67 in IRs/TRs and their poly A sites do not overlap. Rightward oriented gene 68 in IRs and leftward oriented gene 76 in TRs are 3'-coterminal transcripts with the downstream gene 67 (Fig. 2) (Breeden *et al.,* 1992; Holden *et al.,* 1992). For the construction of a gene 67 deletion mutant, 70% of the coding sequence was removed in both copies. The deletion extends from 845 bp downstream of the stop codon of gene 66 to 89 bp downstream of the stop codon of gene 68 in IRs and 680 bp downstream of the stop codon of the gene 76 ORF in TRs which is 135 bp upstream from their 3'-coterminal poly A site (Telford *et al.,* 1992; Breeden *et al.,* 1992). Genes 70, 71 and 72 transcribe in a head to tail pattern. The putative poly A signal of the gene 70 transcript does not overlap with the gene 71 ORF. 66 bp of 3' end of gene 71 ORF overlaps with the 5' end of gene 72 ORF (Fig 2) (Telford *et al.*, 1992). For construction of a deletion mutant in gene 71, 85% of the coding sequence was deleted. The deletion extends from 296 bp downstream of the stop codon of gene 70 within the gene 71 ORF and 510 bp upstream of the start codon of gene 72. Genes 74, 75 and 76 are also transcribed in a head to tail pattern. The 3'-coterminal ploy A site of genes 74 and 75 does not overlap with the gene 76 ORF (Fig. 2). For construction of the deletion mutant in gene 75, 87% of the coding sequence was deleted. The deletion extends from 58 bp downstream of the stop codon of gene 74 to 478 bp upstream of the start codon of gene 76

within the gene 75 ORF. It is clear that the deletion in each target gene sould completely disrupt the gene product but is unlikely to have an effect on the neighbouring genes. With these constructs five mutants in genes 1, 2, 67, 71 and 75 were generated. Restriction enzyme and Southern blot analysis confirmed that the 5 deletion mutants had the expected genome structures without any other detectable differences from wild-type virus. As all the mutants were isolated and purified to homogeneity in non-complementary BHK21/C13 cells, the viability of the mutants demonstrated that genes 1, 2, 67, 71 and 75 are non-essential for virus growth *in vitro.* Analysis of the growth properties showed that the deletion mutants ED1, ED2, ED67 and ED75 were indistinguishable from wild-type virus *in vitro* and that these genes did not independently influence the virus lytic replication cycle in tissue culture; the mutants also did not exhibit a host range phenotype in the tested cell lines or a temperature restriction. In contrast, the deletion mutant in gene 71 grew significantly less well than the wild-type virus and its revertant *in vitro.* The final yield of ED71 was about five to ten-fold lower than that of wild-type virus and it also forms smaller and tighter plaques, compared to that of wild-type virus. The growth properties of the revertant of ED71 were identical to those of wild-type virus as was the yield, plaque size and morphology. The restoration of the wild-type phenotype indicated that deletion of gene 71 is responsible for the defective growth of ED71.

4. 3. EXPRESSION OF VIRAL GENES IN *E. COLI* AND GENERATION OF SPECIFIC ANTISERA AGAINST THE VIRAL GENE PRODUCTS

To identify the products of the five viral genes specific antibodies were raised against portions of the five target genes which had been separately expressed as ß-galactosidase fusion proteins in *E.coli*.. To construct expression plasmids, the parts of the encoding regions of the target genes were separately cloned into pUR vectors to fuse in frame with the 3' end of the ß-galactosidase gene. Restriction enzyme digestion analysis showed that all the constructed recombinants had the expected digestion pattern indicating that the insertions were correct. Sequence analysis confirmed that the inserted coding sequences were fused in frame with the 3'-end of the ß-galactosidase gene. Using the constructed expression plasmids, the gene 67

and 71 fusion proteins were overexpressed as full length fusion proteins with the expected molecular weights from transformed *E. coli* . The gene 1 and 2 fusion proteins were expressed at a high level, but the expressed fusion proteins were partially degraded and proteolysis could not be prevented under modified conditions. The gene 75 fusion protein was expressed at a low level. Expression could not be increased by expression in different host cells or at different temperatures and by induction with different amount of IPTG, which may indicate that the gene 75 fusion protein is toxic to host cells. Cell fractionation experiments demonstrated that all the expressed fusion proteins were present as insoluble inclusion bodies. In general insoluble antigen is better than soluble antigen for raising antibody (Harlow, 1988). As the aim of expression of the target genes was to raise specific antibodies against the gene products, purification of inclusion bodies was chosen for preparation of the antigens.

Since there are individual differences among rabbits in terms of the immune reaction to antigens (Harlow, 1988), each of the fusion proteins was injected into two rabbits. To enhance the antigenicity of the fusion proteins, Freund's adjuvent was used and multiple sites of injection were also chosen to avoid skin damage and to obtain a good immune reaction. As there is little cross-reaction between *E.coli* and mammalian cells, the EHV1 infected cells extracts were directly used as antigens to check the antiserum in Western blotting and immunoprecipitation assays. Two specific antisera were detected from rabbits immunised by fusion proteins, FP67 and FP71 after one initial immunization and four boosts and designated anti-67 and anti-71, respectively. Anti-67 specifically recognised an infected cell polypeptide with M_r of 36KD and anti-71 recognised a 192 KD polypeptide in EHV1 infected cell extracts.

Unfortunately, the fusion proteins FP1, FP2 and FP75 did not induce any detectable specific antibodies against their gene products in the immunised rabbits. Although as stated previously, analysis of the fusion proteins showed that FP75 is not well expressed, compared to the FP67 and FP71 and FP1 and FP2 were partially degraded, the data from Western blotting against the expressed fusion proteins, FP1, FP2 and FP75, showed that a high level of anti-fusion protein antibodies was induced in the antisera. For negative control the cell extracts from deletion

mutant ED1, ED2 and ED75 infected cells were also used as antigen in Western blotting and immunoprecipitation to check the antisera. The results show that that no differences were detected between mutant and wild-type virus infected cells. These observation suggest two possibilities, one is that the products of these genes may be regulated as low abundance proteins and could not be distinguished from mock background bands; another is that these fusion protein did not induce any specific antisera against the gene products as the reation to the fusion protein could be due to anti-β-gal antibodies. However, the reason for the failure in antibody induction is not clear. Since no obvious phenotype was observed from the characterisation of these deletion mutants, no further effort was put into producing antisera against these gene products.

4. 4. IDENTIFICATION AND CHARACTERISATION OF THE EHV1 GENE 67 POLYPEPTIDE.

The sequence analysis predicted that gene 67 in EHV-1 Ab4 encoded a 272 amino acids polypeptide which contains a short putative amino-terminal membrane signal sequence and two N-linked glycosylation sites. It also contains six phosphorylation sites. Sequence analysis also indicated that gene 67 appeared to be unique to equine herpes virus. A homologous gene has been found in EHV-4 (Nagesha *et al* .,1993) and bovine herpesvirus type 1 (Leung-Tack *et al.*, 1994). At the begining of the project, the product of the gene had not been identified and its function was unknown. The construction and characterisation of the gene 67 deletion mutant showed that gene 67 is nonessential for virus growth *in vitro* and deletion of gene 67 does not affect virus growth properties *in vitro.* However, the deletion mutant is somewhat attenuated in a mouse model, which indicate that gene 67 protein may play a important role *in vivo,* particularly in determination of pathogenicity (Tim Fitzmaurice, personal conmunication). To elucidate the function of gene 67, the gene 67 polypeptide was identified and characterised by using a specific rabbit antiserum, anti-67, which was raised against the fusion protein pUEH67. The results showed that anti-67 specifically recognised a polypeptide with a M_r of 36 KD on 10% SDS-PAGE in EHV1 infected cell extracts, but not in ED67 or mock infected cells. The same polypeptide was not recognised by preimmune serum in EHV1 infected cells extracts. This

recognition was specifically inhibited by the fusion protein pUEH67. These results demonstrated that EHV1 gene 67 encodes a 36 KD M_r polypeptide in EHV1 infected cells. To further explore the properties of the gene 67 protein, the protein was characterised in terms of regulation of expression, cellular and virion localization and post-translational modification. The 36 KD protein product of gene 67 is first detectable by one hour post infection, expression is not dependent on viral DNA replication, but it is not synthesised under immediate early conditions. The transcriptional pattern of the genome of EHV-1 is similar to that of HSV-1 (Cohen *et al.*, 1975a ; Gray *et al.*, 1987a, b; Honess & Roizman 1974; Clements *et al.*, 1977). Therefore, in comparison with two HSV-1 genes, an immediate-early gene (IE1) and an early gene (UL42), it was concluded that the gene 67 polypeptide is regulated as an early protein.

Sequence analysis data showed that gene 67 contains two N-linked glycosylation sites and 6 phosphorylation sites throughout the amino acid sequence. Labelling experiments demonstrated that the protein is phosphorylated, but not glycosylated which implies that the predicted N-glycosylation sites are not used. The protein of gene 67 is phosphorylated and in contrast to its cell-associated form which is an abundantly expressed polypeptide the protein is also found in the nucleocapsid/tegument fraction at low abundance. This suggests that gene 67 plays roles in both infected cells and virions.

Sequence analysis showed that a short putative membrane signal sequence is present near the amino terminus of the gene 67 ORF at residues 28-46. This hydrophobic sequence is followed by a signal cleavage-like sequence, which conforms to the -3, -1 rule of Von Heijne (1983, 1986), at residues 43 and 44; however this signal cleavage site is likely to be buried within the ER membrane and may not be utilised. Since other potential membrane spanning sequences are lacking in the protein sequence, the possibility exists that the putative signal sequence serves as a membrane-spanning segment for the gene 67. Cellular fractionation experiments showed that the majority of the 36 KD polypeptide is present in the cellular membrane fraction in the presence of 1 M NaCl and pH 11.5 100 mM-Sodium carbonate buffer, suggesting that the protein is highly associated with cellular membranes. It should be noted that the method used

to fractionate cellular membranes will not separate cytoplasmic virions from membranes. However, this contamination would have little effect on the distribution of the gene 67 protein within cells, because (1) the gene 67 protein is a low abundance virion protein and (2) the majority of EHV1 virions were released into the cell medium at late stage of infection.

An interesting finding is that anti-67 also specifically picks up four other proteins from EHV1 infected cell extracts, but not from mock or ED67 infected cell extracts. The preimmune serum did not recognise these proteins. The expression of these polypeptides was different from each other and also different from that of the 36 KD gene 67 protein. The 33KD polypeptide was expressed in a similar manner as the gene 67 protein (36KD), except at a lower level of expression. The expression patterns of the other three polypeptides were quite different to that of the gene 67 protein. The 26 KD polypeptide was initially expressed at the same time as the 36 KD protein, but the amount of the protein gradually decreased. The 31.5 KD and 29 KD plypeptides appeared at 12 h p.i.and gradually decreased until 24 h p.i. Pulse-chase experiment shows that the 36 KD gene 67 product is not chased into any of these low M_r polypeptides. Since sequence analysis shows that there are five methionine codons downstream of the start codon of the gene 67 protein, these data suggest the possibility that the lower M_r polypeptides could be expressed from these downstream ATGs within the gene 67 expression cassette.

One of the most interesting characteristics of the gene 67 protein is its filament or ribbon-like distribution pattern within the infected cell as detected by immunofluorescence. A similar distribution of the transiently expressed gene 67 protein with pCMV67 was also seen in lipofected cells suggesting that the formation of the filament-like structure by the gene 67 protein is independent of other virus proteins. As far as we are aware, this is a novel pattern not previously described for any other herpes virus polypeptide. The staining arrangement is striking and varies depending on the extent of infection. Specific filaments, or ribbon-like structures confined entirely to the cytoplasm are apparent in both virus infected and lipofected cells. Small rod shaped structures appear to coalesce to form large filaments which become concentrated in a perinuclear location although strands continue to wind through the entire

cytoplasm. This overt configuration suggests that the protein may be associating with some component of the cytoskeleton although the filaments are much thicker than those normally observed on routine staining of cytoskeletal elements (Klymkowsky and karnovsky *et al.*, 1994; DwyerNield *et al.*, 1996). However, staining by double labelling immunofluorescence with anti-67 and anti-tubulin or anti-actin and treatment with tubulin, actin or Golgi inhibitors demonstrated that the protein did not colocalise with either microtubulin or Golgi apparatus. An association with intermediate filaments has not yet been assessed although the gross morphology of the staining pattern is not indicative of intermediate filaments. As a non-glycoprotein, the strong association with membranes requires further analysis for post translational modifications such as myristylation which may lead to membrane binding (Bryant & Ratner 1990).

A gene called IR6, equivalent to strain Ab4 gene 67, has also been found in EHV1 strain Kentucky A. As expected, it has very similar characteristics to those of gene 67. It is transcribed as a 1.2-kb mRNA whose synthesis begins at very early times (1.5 h) after infection and continues throughout infection (Breeden *et al.*, 1992). O'Callaghan *et al.* (1994) have shown that the IR6 gene encodes an approximately 33 KD polypeptide, which is expressed as early as 1 to 2 h postinfection and is synthesised throughout the infection. The IR6 protein is phosphorylated, associates with purified EHV1 virions and nucleocapsids and was also found to be distributed as "dash-shaped" structures that localised to the perinuclear region.

Overall the results from these experiments showed that EHV1 gene 67 encodes a 36 KD phosphoprotein which is regulated as an early protein and accumulates throughout infection, but is a low abundance component of virions. It is associated with cellular membranes and is distributed as ribbon or filament-like structures in the cytoplasm. Although deletion of gene 67 does not affect virus growth in tissue culture, an *in vivo* study has shown that ED67 is attenuated pathogenicity in a mouse model of EHV1 infection (H. J. Field personal communication). In addition, it has been found that the modification or deletion of gene 67 is the only alteration in the genome of the RacH strains (Osterrieder *et al.*, 1994a, 1994b), which

are apathogenic for laboratory animals and the horse, and have been used as a live vaccine against EHV1 infection (Mayr *et al.*, 1968). These mutations include deletion of the gene 67 ORF in both TRs/IRs in RacM36 and amino acid substitutions at positions 34, 42, 110 and 134 in RacL11, RacL22 and RacM24, respectively (Osterrieder *et al.*, 1996a). It is suspected that the substitution at position Leu-134 affects the formation of the ribbon-like structure of the gene 67 protein. Recently, Colle and O'Callaghan (1996) have found that the EHV1 protein kinase (PK) encoded by gene 69 forms a complex with the gene 67 protein, and also colocalised with the gene 67 protein in the cytoplasm as a novel ribbon-like structure. The normal distribution of the gene 67 protein is essential for the localisation of EHV1 PK protein in the cytoplasm and this colocalisation is required for the maximal protein kinase enzyme activity. The Us PKs of HSV1 and PRV have been shown to be involved in virus egress and determination of virulence (Wagenaar *et al.*, 1995; Kimman *et al.*, 1992; Nishiyama *et al.*, 1992 and Kurachi *et al.*, 1993), although they are dispensable for virus growth in tissue culture (Longnecker and Roizman, 1987). These findings suggest that gene 67 may function as a cofactor required for activation of the PK or simply serve as an anchor to retain the EHV1 Us PK protein in the cytoplasm.

Together these features of the gene 67 protein suggest that it could play an important role in the virus replication cycle, particularly in determination of pathogenicity. However its precise role in the virus life-cycle remains unknown.

4. 5. IDENTIFICATION AND CHARACTERISATION OF THE EHV1 GENE 71 POLYPEPTIDE.

EHV-1 gene 71 is located in the short unique region of the genome and is predicted to encode a 797 amino acids polypeptide with a M_r of 80.1K (Telford *et al.*, 1992). The putative gene 71 polypeptide possesses hydrophobic amino and carboxy-terminal domains which may serve as a potential signal sequence and transmembrane domain respectively (Fig.35). It also contains a single potential N-linked glycosylation site and an extensive region encoding a high proportion of serine and threonine residues. These features suggest that the gene 71 product is a class 1 membrane glycoprotein with heavy O-linked and N-linked glycosylation. Similar sequence

features have been found in HSV-1 gC and HSV-2 gG (Serafini-Cessi *et al.*, 1985) which are heavily O-glycosylated and have an extended region containing a high proportion of serine and threonine residues, presumed to contain most of the O-linked glycosylation sites (McGeoch *et al.*, 1987; Serafini-Cessi *et al.*, 1985). HSV1 gC plays a major role in virus adsorption and penetration (Herold, *et al.* 1991) and mutants in HSV2 gG are attenuated pathogenicity in a mouse model (Taha, Ph.D. thesis; Meigneir *et al.*, 1988).

The data on identification and characterisation of the gene 71 protein, presented in this thesis support the predicted features of gene 71 from the sequence analysis. To identify the product of gene 71, the C-terminal portion of the gene 71 encoding sequence was expressed as a ß-galactosidase fusion protein. A specific antiserum, anti-71, was raised from rabbits immunised with this fusion protein. Anti-71 specifically recognises a polypeptide with a M_r of 192 KD in EHV1 infected cell extracts. The reaction between the gene 71 protein and anti-71 is specifically inhibited by the fusion protein pUEH71. The preimmune serum did not recognise this protein in EHV1 infected cell extracts. The polypeptide was not detected in mock and ED71 infected cell extracts, and it is restored in Re71 infected cells. These findings clearly demonstrate that gene 71 encodes a 192KD M_r polypeptide in EHV-1 Ab4 infected cells.

The carbohydrate motifs (either high mannose or complex) of virus glycoproteins are most commonly linked to asparagine residues via the dolicholphosphate pathway and are sensitive to the inhibitory effects of tunicamycin (Leavitt, *et al.*, 1971) and digestion with endo glycosidase F (endoF) (Elder and Alexander, 1982; Maley *et al.*, 1989). It has also been shown that several animal viruses, including herpesviruses, contain tunicamycin resistant O-linked carbohydrates on their envelope glycoproteins (Holmes *et al.*, 1981; Johnson and Spear, 1983; Niemann *et al.*, 1983,1982; Olofsson *et al.*, 1981; Shida and Dales, 1981). These oligosaccharides are sensitive to the treatment with monensin or neuraminidase/O-glycanidases (Carlsen and Pierce, 1972; Bahl and Agarwal, 1969; Li and Li, 1970) and are primarily responsible for the proteins slow electrophoretic mobility in SDS-PAGE (Johnson and Spear, 1983). Sequence analysis showed that the gene 71 protein contains a single putative N-linked glycosylation site and an

extensive region (residues 22-465) encoding a high proportion (87%) of serine and threonine residues which is likely to be heavily O-glycosylated (Telford *et al.,* 1992). As expected, the M_r of the gene 71 poypeptide on SDS-PAGE is much greater than the putative M_r from the amino acid sequence (80.1 KD). This suggests that the gene 71 protein is likely to be modified as a heavily O-glycosylated protein. Post-translational modification experiments confirmed this hypothesis. Firstly the 192 KD gene 71 protein was labelled by ^{14}C glucosamine. The 192 KD gene 71 protein was reduced to a 183 KD polypeptide following treatment with tunicamycin, and the digestion with endo glycosidase F only slightly reduced the M_r of the gene 71 protein. Treatment with monensin or neuraminidase/exoglycanidases dramatically reduced the molecular weight of the gene 71 protein to 80 KD which is same as the molecular weight of the primary product of gene 71. These results confirmed that the gene 71 protein is a glycoprotein with heavy O-linked glycosylation and limited N-linked glycosylation. These findings are consistent with the putative features of the gene 71 protein from sequence analysis. The 80 KD species produced following treatment with either monensin or exoglycanses was also shown to be present at low abundance in total infected cell extracts and the cytosol fraction, but not in mock infected cells. It was not found in ED71 infected cells. These data suggest that it is likely to be the primary product of gene 71.

The putative EHV1 gene 71 protein contains a N-terminal signal sequence of hydrophobic amino acids with one charged side chain between the N-terminus and the hydrophobic region; it also contains a 25 residue hydrophobic region near the C-terminus (Fig. 36). These sequence features suggested that the gene 71 protein is likely to be present on the surface of cell membranes and on virion envelopes as a class I membrane glycoprotein. Cellular fractionation experiments confirmed that the gene 71 polypeptide is strongly associated with cellular membranes. Virion surface labelling experiments further confirmed that the product was located on the surface of virion envelopes. These results demonstrate that the gene 71 protein is a class I membrane glycoprotein which is present on both cellular membranes and virion envelopes.

As a virus structural protein, the gene 71 polypeptide is expected to be regulated as a late protein in infected cells. Transcription and translation of the EHV-1 genes, like those of HSV1, are temporally regulated into an immediate-early, early and late pattern (Cohen *et al.*, 1975a; Caughman *et al.*, 1985; Gray *et al.*, 1987a, b; Clements *et al..*, 1977). A timecourse of expression showed that the gene 71 protein is first detected at 8 h p.i. and its synthesis is significantly reduced by the presence of PAA. In control experiments, the regulation of two HSV1 genes, a true late gene (US11) and an early gene (UL42), was examined and shown to have the expected patterns. In comparison, it was concluded that the gene 71 protein is regulated as a leaky late polypeptide.

Overall these results demonstrate that the EHV1 gene 71 encodes a typical class I membrane glycoprotein which is heavily O-glycosylated with a M_r 192KD in infected cells and a M_r over 200 KD in virion envelopes. It is regulated as a leaky late polypeptide.

4. 6. DETERMINATION OF THE RELATIONSHIP BETWEEN THE EHV1 GLYCOPROTEIN GP300 AND THE GENE 71 POLYPEPTIDE.

Among the identified EHV-1 glycoproteins (Abodeely *et al..*, 1971; Kemp *et al..*, 1974; Perdue *et al.*, 1974; Turtinen and Allen, 1982; Meredith *et al..*, 1989), one with a M_r in excess of 200K, designated either gp1, gp2, gp1/2 or gp300 was first observed in purified virions and mapped to an EcoRI fragment of the genome between map units 0.279 to 0.344 (Allen & Yeargan, 1987). It was subsequently found to be sensitive to monensin and exoglycanases which indicated that gp300 was modified as a heavily O-linked glycoprotein. It was also found to be a virion surface protein with a M_r estimated as 400KD on SDS-PAGE cross-linked with DATD (Whittaker *et al.*,1990). Whittaker *et al.*, (1992a) appeared to demonstrate that gp300 was encoded by EHV-1 gene 28 using an anti-gp300 monoclonal antibody, P19, and a fusion protein composed of the portion of the protein encoded by codons 107 to 504 of gene 28 fused to the 3' end of glutathione S-transferase (GST) . They found that MAb P19 recognised a 70 KD species corresponding to the expected size of the fusion protein from the recombinant

fusion protein expression plasmid transformed cell extract. This 70 KD species was not present in cells transformed with the parent plasmid and P19 did not recognise GST. From these findings, the authors concluded that EHV1 gene 28 encodes the high M_r glycoprotein gp300.

During the course of this study it became apparent that the EHV1 gene 71 polypeptide had very similar characteristics to the EHV1 gp300. It was reasonable therefore to raise the question whether the two proteins are identical and if so which gene encoded it. From the amino acid sequence feature, EHV1 gene 28 did not appear to be a typical membrane glycoprotein modified with heavy O-linked glycosylation. It has no apparent signal sequence (McGeoch, 1985) and there is only one region (amino acids 130-148) of sufficient length and hydrophobicity which could be predicted to be membrane spanning (Kyte and Doolittle, 1982). Furthermore, unlike gene 71 there is no region rich in serine or threonine residues which could serve as O-linked glycosylation sites in the gene 28 amino acid sequence.

To resolve the anomaly, polypeptides from EHV1 wild-type virus, ED71 and Re71 infected cells were compared under the same SDS-PAGE conditions with both antibodies, anti-71 which is specific to the gene 71 protein and P19 which was used to identify gp300 (Whittaker *et al.*, 1992a). Comparative experiments demonstrated that the gene 71 polypeptide and gp300 synthesised in EHV-1 Ab4 infected cells had identical electrophoretic mobilities to each other on both SDS-PAGE cross-linked either with bisacrylamide or DATD in the presence or absence of tunicamycin. Both gp300 and the gene 71 protein were absent in cells infected with the mutant ED71 in which the gene 71 ORF was deleted, but were detected in cells infected with the revertant Re71 in which the gene 71 ORF has been restored. This restoration of the gene 71 protein in Re-71 ruled out the possibility of a secondary mutation in ED71 which could have disrupted gene 28. These results demonstrated therefore that (1) gp300 is the same polypeptide as the gene 71 product and (2) is actually encoded by EHV-1 gene 71 and not by gene 28 as previously determined by Whittaker *et al.* (1992a).

It has recently been found that none of the monoclonal antibodies (MAbs) made against gp300 specifically recognise the transiently expressed EHV1 gene 28 gene protein product in RK13 cells from a gene 28 expression vector, but most of these MAbs can specifically recognise the EHV1 gene 71 protein product transiently expressed by a gene 71 expression vector (D. M. Meredith, personal communication). These data is in agreement with our conclusion that EHV1 gp300 is the same polypeptide as the gene 71 protein and is encoded by gene 71.

4. 7. INVESTIGATION OF THE ROLE OF GENE 71 IN THE VIRUS LIFE-CYCLE.

Herpesvirus membrane proteins have been found to be involved in virus entry, egress and transmission. Mutants in HSV1 and other alphaherpesvirus genes which encode gD, gH, gL, and the UL11 and UL20 proteins have been shown to be defective in adsorption, penetration and egress, and appear to be responsible for different steps of the process of virus entry and release (Baines *et al.*, 1991; Capadelli-Fiume *et. al.*, 1991; Desai *et al.*, 1988; Baines and Roizman, 1992; Hutchinson *et al.*, 1992a; Roop *et al.*, 1993; Rauh and Mettenleiter, 1991a, b; Peeters, 1992a.b).

EHV1 strain Ab4 gene 71 has no known homologue in other herpesviruses, except a positional counterpart, US5, in HSV1 (Telford *et. al.*, 1992). It has been demonstrated that gene 71 encodes a large (M_r 192kD) heavily O-linked membrane glycoprotein which is associated with cellular membranes and the virion envelope. Sequence analysis suggested that the serine and throenine-rich region (amino acids 22 to 465) in the protein may form highly extended semi-flexible rods of 71 nm in length on the virion surface (McGeoch *et. al.*, 1993). As a typical class I membrane glycoprotein, the gene 71 product could possibly play a role in virus entry into cells, egress from cells or transmission from cell to cell. It has been demonstrated that the gene 71 negative mutant, ED71, has defective growth and produces smaller and tighter plaques, compared to that of wild-type virus and its revertant. The *in vivo* study showed that ED71 was somewhat attenuated in a mouse model of pathogenicity (H.J. Field, personal communication). These

phenotypes suggested that the gene 71 protein may play an important role in the virus life cycle and in determination of pathogenesis.

To investigate the role of gene 71 in the virus life cycle, the efficiency of entry into cells, egress from cells and transmission from cell to cell of ED71 was examined *in vitro* and compared with wild-type virus and its revertant. The adsorption/penetration assays demonstrated that ED71 is defective in both adsorption and penetration. This finding supports the predicted features of the gene 71 protein as a long protruding membrane glycoprotein on virion envelopes (McGeoch *et. al.*, 1993). As the defect in adsorption may also make virus vulnerable to acid buffer, we could not rule out the possibility that the demonstrated defect in the efficiency of virus adsorption could consequently result in a decrease in penetration.

It was found that purified ED71 virions display a 10 -20 fold higher particle/pfu ratio than that of wild-type virus and revertant which suggested that the deletion may effect virion maturation. Quantitation of progeny virus showed that the release of infectious virus from ED71 infected cells was decreased by 100 fold and significantly delayed. The yield of cell-associated infectious virus from ED71 infected cells is also decreased about 2-3 fold, compared to that of wild-type virus and revertant. E.M. analysis of virion distribution showed that (1) The percentage of capsids in the nuclei of ED71 infected cells is much higher than that in wild-type virus and revertant infected cells. (2) The amount of particles (enveloped and unenveloped capsids) in the cytoplasm of ED71 infected cells is much less than in wild-type virus and revertant infected cells and the ratio of enveloped to unenveloped ED71 capsids in the cytoplasm is lower than that of wild-type and revertant. (3) The percentage of extracellular virions in ED71 infected cells is less than that in wild-type infected cells. These observations indicated that deletion of gene 71 impairs nucleocapsid maturation and virion release from infected cells suggesting that the gene 71 protein plays a role in virus maturation and egress. These defects could also be the reason for defective growth of ED71 and the higher particle/pfu ratio of purified ED71 virions.

As a result of the defect in adsorption/penetration and release, it seemed likely that transmission via release and re adsorption would be affected. Since in the presence of appropriate amounts of neutralising antiserum, the virus transmission from cell to cell via the medium can be blocked *in vitro,* the effect of neutralizing antiserum on plaque size was examined. The data show that in the absence of neutralising antiserum the plaques formed by ED71 are contained and smaller than those formed by wild-type virus. In the presence of neutralising antiserum, the size of plaques formed by ED71 is not significantly reduced, while the size of those formed by wild-type virus and revertant are markedly reduced and become similar to those formed by ED71. These data demonstrated that the plaques formed by wild-type virus and revertant are mediated by transmission via medium and direct cell to cell transmission, while those formed by ED71 (smaller and contained) are only mediated by direct cell to cell spread indicating that transmission of ED71 via the medium is greatly impaired. When low m.o.i. growth was carried out in the presence of neutralising serum the yield of ED71 was only reduced 5-10 fold, but the yield of EHV1 and Re71 was reduced 80-100 fold, resulting in little difference in yield between ED71 and EHV1/RE71, compared to the 10-20 fold difference in the absence of neutralising serum. This again is in agreement with the main mode of spread of ED71 *in vitro* being via direct cell to cell transmission, compared to that of wild-type virus being spread via both direct cell to cell and the medium. These results confirmed the hypothesis that the defect in virus release and readsorption consequentially affects virus transmission.

Interestingly, at low m.o.i. the yield of infectious virus from ED71 infected cells before 24 h is about 10 fold higher than that from wild-type virus and revertant infected cells, and this increase is not reduced by the presence of a neutralising antiserum. The same increase is not clearly seen following a high m.o.i. Electron microscopy analysis showed that very similar numbers of particles were produced from ED71, wild-type and revertant infected cells. This pattern of infection implies that gene 71 protein may indirectly enhance virion de-envelopment or transport to the nucleus, leading to a more rapid decline in input titre and production of progeny virus. Alternatively it may be due to the delayed/impaired release of ED71 leading to a build up of infectious virus in the cells. However, at the moment the mechanism of this increase is not

clear.

Overall these studies show that deletion of gene 71 impairs virus egress from cells which indicates that the gene 71 protein plays a role in virus maturation and egress. Deletion of the gene 71 protein also decreases virus entry into cells. At this stage it is reasonable to assume that this is the result of the predicted semi-flexible spikes being absent from the virus surface. The deletion has a consequential role in the efficiency of virus transmission from infected cell to uninfected cell, and the mode of infection. These defects could account for the virus attenuation *in vivo* (H. J. Field, personal communication). However, the molecular mechanism by which the gene 71 protein performs its function(s) in the virus life cycle is still unclear.

It should be noted that the 3' terminal 80 % of the gene 71 ORF is deleted in ED71 and the gene 71 product is not detected by a specific antiserum (anti-71) and a monoclonal antibody (P19) (Sun *et al.*, 1994). The ß-galactosidase expressed from the lacZ insertion in gene 71 ORF should not affect virus behaviour in virus entry into cells and release from cells since a deletion mutant with a similar *lacZ* substitution in EHV1 gene 75 ORF (Sun and Brown, 1994) does not show any defect in virus growth and entry into cells, egress from cells and transmission from cell to cell (Section 3.4 and data not shown).

4. 8 FUTURE PROSPECTS

Characterisation of ED71 demonstrated that the gene 71 protein plays multiple roles in the virus life cycle such as mediation of virus egress from cells and virus entry into cells. Subsequently it plays a role in virus transmission. ED71 has also been demonstrated to be attenuated *in vivo*. Sequence analysis showed that the gene 71 protein is a class I membrane glycoprotein. It contains N- and C-terminal hydrophobic regions which serve as signal sequence and transmembrane domains. It also contains an extended region with a high proportion of serine and threonine residues, presumed to contain most of the O-linked glycosylation sites (McGeoch *et al.*, 1987; Serufini-Cessi *et al.*, 1985). It has been suggested if the serine and threonine rich region is heavily O-glycosylated it could form a highly extended semi-flexible rod of 70nm in length on the surface of the virus particle (McGeoch *et al.*, 1993). Some reports about the possible functions of O-linked oligosaccharides suggest that they could play a role in polypeptides acquiring their final conformation (Gibson *et al.*, 1980), in increasing hydration of cells, in protecting virus from degradation or in transport of viral glycoproteins or virions to the cell surface (Johnson and Spear, 1983). These data suggest that the gene 71 protein is involved in different stages of virus life cycle and contains different domains which could be the molecular basis of its function. Characterisation of these domains will provide essential information for further understanding the functions of the protein, for example use of antibodies to specific domains of the protein in conjunction with electron microscopy to reveal the precise arrangement of the gene 71 polypeptide such as the semi-flexible rod structure formed by the O-glycosylated serine and threonine rich region on the virion envelope and construction and characterisation of deletion mutants in which the different domains of the gene 71 protein such as the serine and threonine rich region and N-terminal or C-terminal hydrophobic domains of the gene 71 protein.

Characterisation of the gene 67 protein demonstrated that it is expressed as a very abundant early protein during the virus lytic infection. The gene 67 protein is modified as a phosphoprotein and distributed as a novel filament like structure in the cytoplasm. Although

the gene 67 protein is nonessential for virus growth *in vitro,* the gene 67 deletion mutant is attenuated. These data indicate that the gene 67 protein could play role in the determination of virus pathogenicity. To address the role of the gene 67 protein it will be necessary to determine the subcellular location of the protein using immune electron microscopy plus different antibodies against specific cellular components or organelles. Construction of different length of truncated forms of the gene 67 protein, to determine the essential amino acid sequence requirements for the normal processing and distribution of the protein, will also provide important data for understanding the function of the protein. In addition, further characterisation of the gene 67 deletion mutant *in vivo*, particularly in the natural host, compared with wild-type and revertant is essential for elucidating the role of the protein in pathogenicity.

REFERENCES

ABLASHI, D. V., SALAHUDDIN, S. Z., JOSEPHS, S. F., IMAM, F., LUSSO, P., GALLO, R. C., HUNG, C., LEMP, J., AND MARKHAM, P. D. (1987). HBLV (or HHV-6) in human cell lines. Nature, **329**, 207.

ABODEELY, R. A., PALMER. E. AND LAWSON. L. A., AND RANDALL, C. C. (1971). The proteins of enveloped and de-enveloped equine abortion (herpes) virus and the separated envelope. Virol. **44,** , 146-152.

ABODEELY, R. A., LAWSON, L. A., AND RANDALL,.C. C. (1970). Morphology and entry of enveloped and de-enveloped equine abortion (herpes) virus. J. Virol. **5,** 513-523.

ACE, C. I., DALRYMPLE, M. A., RAMSAY, F. H., PRESTON, V. G., AND PRESTON, C. M. (1988). Mutational analysis of the herpes simplex virus type 1 transinducing factor Vmw65 J. Gen. Virol. **62**, 2595-2605.

ADDISON, C., RIXON, F. J., PALFREYMAN, J. W., O'HARA, M., AND PRESTON, V. G., (1984). Characterisation of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. Virology **138,** 246-259.

AGIUS, C. T., AND STUDDERT, M. J. (1994). Equine herpesviruses 2 and 5: Comparisons with other members of the subfamily gammaherpesvirinae. Advances in Virus Research, **44** 357-378.

ALLEN, G. P., AND COOGLE, L. D. (1988). Characterisation of an equine herpesvirus type 1 gene encoding a glycoprotein (gp13) with homology to herpes simplex virus glycoprotein C. J. Virol. **62,** 2850-2858.

ALLEN, G. P., AND YEARGAN, M. R. (1987). Use of λgtII and monoclonal antibodies to map the genes for six major glycoproteins of equine herpesvirus type 1. J. Virol. **61,** 2454-2461.

ALLEN, G. P., AND BRYANS, J. T. (1986). Molecular epizootiology, pathogenesis and prophylaxsis of equine herpesvirus-1 infections. In " Veterinary microbiology- Molecular and Clinical Perspectives", pp. 78-144. Edited by R. Pandey. Progress in Veterinary Microbiology and Immunology, vol. **2,** S. Karger, Basel.

ALLEN, G. P., YEARGAN, M. R., TURTINEN, L. W., BRYANS, J. T., AND MCCOLLUM, W. H. (1983). Molecular epizootiologic studies of equine herpesvirus-1 infections by a restriction endonuclease finger printing of viral DNA. Am. J. Vet. Res. **44**, 263-271.

ALLEN, G. P., AND TURTINEN, L. W. (1982). Assessment of the base sequence homology between the two subtypes of equine herpesvirus-1. J. Virol. **44**, 249-255.

ALLEN, G. P., MCGOWAN, J. J., RANDALL, C. C., MANCINI, W., CHENG, Y.-C., AND GENTRY, G. A. (1979). Purification and characterization of deoxythymidine kinase (dTK) induced and dTK-373 mouse cells by Equine herpesvirus type 1 (EHV1). Virol. **92**, 367-374.

ALLEN, G. P., COHEN, J. C., RANDALL, C. C., AND O'CALLAGHAN, D. J. (1978 a). Replication of equine herpesvirus type 1 and type 3 : resistance to hydroxyurea and thymidine. Intervirology **9,** 276-258.

ALLEN, G. P., MCGOWAN, J.J., BRYANS, J. T., RANDALL, C. C., AND GENTRY, G. A. (1978 b). Induction of deoxythymidine kinase activity in several mammalian cell lines after infection with six different strains of equine herpesvirus type 1 (EHV1). Virol **90**, 351-359.

ALLEN, G. P., O'CALLAGHAN, D. J., AND RANDALL, C. C. (1977). Purification and characterisation of equine herpesvirus induced DNA-polymerase. Vriol. **76**, 395-408.

ANDERSON, K., AND GOODPASTURE, E. W. (1942). Infection of newborn Syrian hamsters with the virus of mare abortion. American Journal of Pathology. **18**, 555-561.

ARON, G. M., PURIFOY, D. J. M., AND SCHAFFER, P. A. (1975). DNA synthesis and DNA polymerase activity of herpes simplex virus type 1 temperature-sensitive mutants. J. Virol. **16**, 498-507.

ARSENAKIS, M., CAMPADELLI-FIUME, G., AND ROIZMAN, B. (1988). Regulation of glycoprotein D synthesis: does alpha 4, the major regulatory protein of herpes simplex virus 1, regulate late genes both positively and negatively? J. Virol. 62 148-158.

ASWELL, J. F., ALLEN, G. P., JAMIESON, A. T., CAMPBELL, D. E., AND GENTRY, G. A. (1977). Antiviral activity of arabinosylthymine in herpesviral replication: mechanism of action *in vivo* and *in vitro.* Antimicrob. Ag. Chemother. **12,** 243-254.

AUDONNET, J-C., WINSLOW, J., ALLEN, G. P., AND PAOLETTI, E. (1990). Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI, and gE. J. Gen. Virol. **71**, 2969-2978.

AVITABILE, E., GAETA, S. D., TORRISI, M. R., WARD, P. L., ROIZMAN, B., AND CAMPADELLI-FIUME, G. (1995). Redistribution of microtubules and Golgi apparatus in herpes simplex virus-infected cells and their role in viral exocytosis. J. Virol. **69** 7472-7482.

AVITABILE, E., WARD, P.L., LAZZARO, C. D., TORRISI, M. R., ROIZMAN, B., AND CAMPADELLI-FIUME, G. (1994). The herpes simplex virus UL20 protein compensates for the differential disruption of exocytosis of virions and viral membrane glycoproteins associated with fragmentation of the Golgi apparatus. J. Virol. **68,** 7397-7405.

AWAN, R. A., CHONG, Y.-C., AND FIELD, H. J. (1990). The pathogenesis of equine herpesvirus type 1 in the mouse: a new model for studying host responses to the infection. J. Gen. Virol. **71,** 1131-1140.

AZMI, M., AND FIELD, H. J. (1993). Cell-mediated antiviral response to equine herpesvirus type 1 demonstrated in a murine infection model by means of adoptive transfer of immune cells. J. Gen. Virol. **74**, 275-280.

BACCHETTI, S., EVELEGH, M. J., AND MUIRHEAD, B. (1986). Identification and separation of the two subunits of the herpes simplex virus ribonucleotide reductase. J. Virol. **57,** 1177-1181.

BAGUST, T. J., PASCOE, R. R., AND HARDEN, T. J. (1972). Studies on equine herpesviruses III. The incidence in Queensland of three different equine herpesvirus infections. Aust. Vet. J. **48,** 47-53.

BAHL, O. P., AND AGRAWAL, K. M. L. (1969). Purification and characterisation of ß-galactosidases and ß-N-glucosaminidase. J. Biol. Chem. **244**, 2970-2970.

BAINES, J. D., AND ROIZMAN, B. (1992). The UL11 gene of herpes simplex virus encodes a function that facilitates nucleocapsid envelopment and egress from cells. J. Virol. **66,** 5168-5174.

BAINES, J. D., WARD, P. L., CAMPADELLI-FIUME, G., AND ROIZMAN, B. (1991). The UL20 gene of herpes simplex virus type 1 encodes a function necessary for viral egress. J. Virol. **65,** 6414-6424.

BARAHONA, H., MELENDEZ, L. V., AND MELNICK, J. L. (1974). A compendium of herpesviruses isolated from non-human primates. Intervirology **3,** 175-192.

BATCHELOR, A. H., AND O' HARE, P. (1990) Regulation of cell type specific activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1. J. Virol. **64,** 3269-3279.

BATTERSON, W., AND ROIZMAN, B. (1983). Characterisation of the herpes simplex virion-associated factor responsible for the induction of alpha-genes. J. Virol. **46**, 371-377.

BAUCKE, R., AND SPEAR, P. G. (1979). Membrane proteins specified by herpes simplex viruses V. Identification of an Fc-binding glycoprotein. J. Virol. **32**, 779-789.

BAUMANN, R. P., RAMANA, V., YALAMANCHILI, V. R. R., AND O'CALLAGHAN, D. J. (1989). Functional mapping and DNA sequence of an equine herpesvirus 1 origin of replication. J. Virol. **63,** 1275-1283.

BAXI, M. K., EFSTATHIOU, S., LAWRENCE, G., WHALLEY, J. M., SLATER, J. D., AND FIELD, H. J. (1995). The detection of latency-associated transcripts of equine herpesvirus 1 in ganglionic neruons. J. Gen. Virol. **76,** 3113-3118.

BAYLISS, G. J., MARSDEN, H. S., AND HAY, J. (1975). Herpes simplex virus proteins: DNA-binding protein in infected cells and in the virus structure. Virol. **68,** 124-134.

BEN-PORAT, T., AND KAPLAN, A. S. (1972). Studies on the biogenesis of herpesvirus envelope. Nature (London). **235,** 165-166.

BERGER, S. L., CRESS, W. D., CRESS, A., TRIEZENBERG, S. L., AND GUARENTE, L. (1990). Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. Cell **61,** 1199-1208.

BISHOP, G. A., MARLIN, S. D., SCHWARTZ, S. A., AND GLORIOSO, J. C. (1984). Human natural killer cell recognition of herpes simplex type 1 glycoproteins: specificity analysis with the use of monoclonal antibodies and antigenic variants. J. Immunol. **133,** 2206-2214.

BISHOP, G. A., GLORIOSO, J. C., AND SCHWARTZ, S. A. (1983). Relationship between expression of herpes simplex virus glycoproteins and susceptibility of target cells to human natural killer activity. J. Exp. Med, **157,** 1544-1561.

BLANTON, R. A., AND TEVETHIA, M. J. (1981). Immuoprecipitation of virus-specific immediate-early and early polypeptides from cells lytically infected with human Cytomegalovirus strain AD169. Virology, **112,** 262-273.

BLEWETT, E. L., AND MISRA, V. (1991). Cleavage of the bovine herpesvirus glycoprotein B is not essential for its function. J Gen Virol **72,** 2083-2090.

BLOCK, T. M., SPIVACK, J. G., STEINER, I., DESHMANE, S., MCINTOSH, M. T., LIRETTE, R. P., AND FRASER, N. W. (1990). A herpes simplex virus type 1 latency-associated transcript mutant reactivates with normal kinetics from latent infection. J. Virol. **64,** 3417-3426.

BOOY, F. P., NEWCOMB, W. W., TRUS, B. L., BROWN, J. C., BAKER, T. S., AND STEVEN, A. C. (1991). Liquid-crystalline, phage-like packing of encapsidated DNA in herpes simplex virus. Cell **64,** 1007-1015.

BORGEN, H. C., AND LUDWIG, H. (1974). Equine herpesvirus 1: Biological and biophysical comparison of two viruses from different clinical entities. Intervirology **4,** 189-198.

BRACKEN, E. C., AND RANDALL, C. C. (1957). Studies on hepatitis in hamsters infected with equine abortion virus. II. Changes in protein, nucleic acid and weight of isolated hepatic nuclei. Am. J. Pathol. **33**, 1009-1022.

BREEDEN, C. A., YALAMANCHILI, R. R., COLLE, C. F., AND O'CALLAGHAN, D. J. (1992). Identification and transcriptional mapping of genes encoded at the Ir/Us junction of equine herpesvirus type 1. Virol. **191,** 649-660.

BROCKMAN, W. W., ACE, T. H., AND NATHANS, D. (1974). characterization of clonal evolutionary variants of SV40 . Cold Spring Harbor symp. Quant. Biol. **39**, 119-127.

BROWN, S. M., MACLEAN, A. R., AITKEN, J. D., AND HARLAND, J. (1994). ICP 34.5 influences herpes simplex virus type 1 maturation and egress from infected cells *in vitro*. J. Gen. Virol. **75,** 3679-3686.

BROWN, S. M., HARLAND, J., AND SUBAK-SHARPE, J. H. (1984). Isolation of restriction endonuclease site deletion mutants of herpes simplex virus. J. Gen. Virol. **65**, 1053-1068.

BROWN, S. M., RITCHIE, D. A., AND SUBAK-SHARPE, J. H. (1973). Genetic studies with herpes simplex virus type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. J. Gen. Virol. **18**, 329-346.

BROWNING, G. F., BULACH, D. M., FICORELLI, N., ROY, E. A., THORP, B. A., AND STUDDERT, M.J. (1988). Latency of equine herpesvirus 4 (equine rhinopneumotitis virus). Vet. Record **123**, 518-519.

BROWNING, G. F., AND STUDDERT, M. J. (1987). Genome heterogeneity of equine betaherpesviruses. J. Gen. Virol. **68,** 1441-1447.

BRUNETTI, C. R., BURKE, R. L., KORNFELD, S., GREGORY, W., MASIARZ, F. R., DINGWELL, K. S., AND JOHNSON, D. C. (1994). Herpes simplex virus glycoprotein D acquires mannose 6-phosphate residues and binds to mannose 6-phosphate receptors. J. Biol. Chem. **269**: 17067-17074.

BRYANT, M., AND RATNER, L. (1990). Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc. Natl. Acad. Sci. USA **87,** 523-527.

BRYANS, J. T., AND ALLEN, G. P. (1989). Herpesviral diseases of the horse In: Herpes diseases of cattle, horses and pigs. eds. by G. Wittmann. Kluwer Acad. Pub. (London).

BRYANS, J. T., AND ALLEN, G. P. (1982). In: Proc 17th Intl. Cong. Herpesvirus Man and Amin. Stnd. Immunol. Proc. Lyon 1981, Dev. Biol. Stnds. **52 :** 495-498.

BRYANS, J. T. (1978). Immunization of pregnant mares with an inactivated equine herpesvirus 1 vaccine. In: Proc. 4th Int. Equ. Inf. Dis. pp 83-92, Ed. by J.T. Bryans and H. Gerber, Vet. Publ. Inc., Princeton, New Jersey, USA.

BRYANS, J. T., and ALLEN, G. P. (1973). *In vitro and in vivo* studies of equine coital exanthema. In: Proc. 3rd Int. Conf. Eq. Inf. Dis., Paris. 1972 (Eds. J. T. Bryans and H. Gerber), Karger, Basel. , pp. 322-336.

BRYANS, J. T., and PRICKETT, M. E. (1969). A consideration of the pathogenesis of abortigenic disease caused by equine herpesvirus 1. In : Proc. 2nd Int. Conf. Eq. Inf. Dis., pp. 34-40. Paris, 1969. Eds. J. T. Bryans and H. Gerber, Karger Basel.

BRYANS, J. T., (1969). On immunity to disease caused by equine herpesvirus-1. J. Am. Vet. Med. Assoc. **155,** 294-300.

BUCKMASTER, E. A., GOMPELS, V., AND MINSON, A. (1984). Characterisation and physical mapping of HSV-1 glycoprotein of approximately $115x10^3$ molecular weight. Virol. **139**, 408-413.

BURKI, F., ROSSMANITH, W., NOWOTNY, N., PALLAN, C., MOSTL, K., AND LUSSY, F. (1990). Viraemia and abortions are not prevented by two commercial equine herpesvirus-1 vaccines after experimental challenge of horses. Veterinary Quarterly **12,** 80-86.

BURROWS, R., GOODRIDGE, D., AND DENYER, M. S. (1984). Trials on an inactivate equid herpesvirus 1 vaccine: challenge with a subtype-1 virus. Veterinary Record **114,** 369-374.

BURROWS, R., AND GOODRIDGE, D. (1984). Studies of persistent and latent equine herpesvirus-1 and herpesivrus-3 infections in the Pirbright pony herd. In: Latent herpesvirus infections in veterinary medicine pp. 307-319. (Ed. G. Wittmann, R. M. Gaskell and H. J. Rziha) Nijhoff, Boston.

BURROWS, R., AND GOODRIDGE, P. (1979). Equine herpesvirus 1 (EHV1): some observations on the epizootiology of infection and on the innocuity testing of live virus vaccines In: Proc. 24th Ann Conf. Am. Assoc. Equine practi., St. Louis pp. 17-28.

BURROWS, R., AND GOODRIDGE, D. (1973). *In vivo* and *in vitro* studies of equine rhinopneumonitis virus strains, In: Pro. of 3rd Int. Conf. Equine Inf. Dis. pp. 306-321. Edited by J. T. Bryans and H. Gerber. Karger, Basle.

BUSH, M., YAGER, D. R., GAO, M., WEISSHART, K., MARCY, A. I., COEN, D. M., AND KNIPE, D. M. (1991). Correct intranuclear localization of herpes simplex virus DNA polymerase requires the viral ICP8 DNA-binding protein. J. Virol **65**, 1082-1089.

CAI, W., GU, B., AND PERSON, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol **62**, 2596-2604.

CALDER, J. M., STOW, E. C., AND STOW, N. D. (1992). On the cellular localisation of the components of the herpes simplex virus type 1 helicase-primase complex and the viral origin-binding protein. J. Gen. Virol. **73,** 531-518.

CAMPADELLI-FIUME, G., FARABEGOLI , F., GAETA, S. D., AND ROIZMAN, B. (1991). Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. J. Virol. **65:** 1589-1595.

CAMPADELLI-FIUME, G., QI, S., AVITABILE, E., FOà-TOMASI, L., BRANDIMARTI, R., AND ROIZMAN, B. (1990). Glycoprotein D of herpes simplex virus encodes a domain which precludes penetration of cells expressing the glycoprotein by superinfecting herpes simplex virus. J. Virol. **64,** 6070-6079.

CAMPADELLI-FIUME, G., ARSENAKIS, M., FARABEGOLI, F., AND ROIZMAN, B. (1988). Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. J. Virol. **62**: 159-167.

CAMPADELLI-FIUME, G., POLETTI, L., DALL'OLIO, F., ANDSERAFINI-CESSI, F. (1982). Infectivity and glycoprotein processing of herpes simplex virus type 1 grown in a ricin-resistant cell line deficeint in N-acetlylglucosaminyl transferase 1. J. Virol. **43**, 1061-1071.

CAMPBELL, M. E. M., PALFREYMAN, J. W., AND PRESTON, C. M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. **180**, 1-19.

CARLSEN, R. B., AND PIERCE, J. G. (1972). Purification and Properties of an α-L-fucosidase from rat epididymis. J. Biol. Chem. **247**, 23.

CARTON, C. A., AND KILBOURNE, E. D. (1952). Activition of latent herpes simplex virus by trigeminal sensory-root section. N. Engl. J. Med. **246,** 172-176.

CAUGHMAN, G. B., ROBERTSON, A. T., GRAY, W. L., SULLIVAN, D. C., AND O'CALLAGHAN, D. J. (1988). Characterization of equine herpesvirus type 1 immediate early proteins. Virol. **163,** 563-571.

CAUGHMAN, G. B., STACZEK, J., AND O'CALLAGHAN, D. J. (1985). Equine herpesvirus type 1 infected cell polypeptides: Evidence for immediate early/early/late regulation of viral gene expression. Virol. **145**, 49-61.

CHANG, Y., CESARMAN, E., PESSIN, M. S., LEE, F., CULPEPPER, J., KNOWLES, D. M., AND MOORE, P. S. (1994). Identification of herpesvirus-like sequences in AIDS-associated Kaposi's sarcoma. Science **266**, 1865-1869.

CHARLTON, K. M., MITCHELL, D., GIRARD, A., AND CORNER, A. H. (1976). Meningoencephalomyelitis in horses associated with equine herpesvirus 1 infection. Vet. Pathol. **13,** 59-68.

CHASE, C. C. L., CARTER-ALLEN, K., LOHFF, C., AND LETCHWORTH III, G. J. (1990). Bovine cells expressing bovine herpesvirus 1 (BHV-1) glycoprotein IV resist infection by BHV-1, herpes simplex virus, and pseudorabies virus. J . Virol **64,** 4866-4872.

CHONG, Y. C., DUFFUS, W. P. H., FIELD, H. J., GRAY, D. A., AWAN, A. R., O'BRIEN, M. A., AND LUNN, D. P. (1991). The raising of equine colostrum-deprived foals: maintenance and assessment of specific pathogen (EHV-1/4) free status. Equine Vet. J. **23,** 111-115.

CHOWDHURY, S. L., BUHK, H-J., LUDWIG, H., AND HAMMERSCHMIDT, W. (1990). Genomic termini of equine herpesvirus type 1. J. Virol. **64**, 873-880.

CHURCHILL, A. E., AND BIGGS, P. M. (1968). Herpes type-virus isolated in cell culture from tumours of chickens with Marek's disease. II. studies *in vivo.* J. Natl. Cancer Inst. **41**, 951-956.

CHUNG,C. T., AND MILLER, R. H. (1988). A rapid and convenient method for the preparation and storage of competentbacterial cells. Nucl. Aci. Res. **16,** 3580.

CLAESSON-WELSH, L., AND SPEAR, P. G. (1986). Oligomerization of herpes simplex virus glycoprotein B. J. Virol. **60**, 803-806.

CLEMENTS, J. B., WATSON, R. J., AND WILKIE, N. M. (1977). Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell **12**, 275-285.

COULTER, L. J., MOSS, H. W. M., LANG, J., AND MCGEOCH, D. J. (1993). A mutant of herpes simplex virus type 1 in which UL13 protein kinase gene is disrupted. J. Gen. Virol. **74,** 387-395.

COGGINS, L. (1979). Viral respiratory disease of horses. Vet. Clin. North Amer. : Lg Anim. Pract. **1**, 59-72.

COHEN, J. C., PERDUE, M. L., RANDALL, C. C., AND O'CALLAGHAN, D. J. (1975 a). Replication of equine herpesvirus type 1: resistance to hydroxyurea. Virol. **67,** 56-67.

COHEN, J. C., RANDALL, C. C., AND O'CALLAGHAN, D. J. (1975 b). Transcription of equine herpesvirus type 1: Evidence for classes of transcript differing in abundance. Virol. **68,** 561-565.

COLLE, C. F., AND O'CALLAGHAN, D. J. (1996). Localization of the Us protein kinase of equine herpesvirus type 1 is affected by the cytoplasmic structures formed by the novel IR6 protein. Virology **220**, 424-435.

COLLE, C. F., FLOWERS, C. C., AND O'CALLAGHAN, D. J. (1992). Open reading frames encoding a protein kinase, homolog of glycoprotein gX of pseudorabies virus, and a novel glycoprotein map within the unique short segment of equine herpesvirus virus type 1. Virology **188,** 545-557.

COOK, M. L., BASTONE, V. B., AND STEVENS, J. G. (1974). Evidence that neurons harbour latent herpes simplex virus. Infect. Immun. **9,** 946-951.

CORDINGLEY, M. G., CAMPBELL, M. E. M., AND PRESTON, C. M. (1983). Functional analysis of a herpes simplex virus type 1 promoter: Identification of upstream regulatory sequences. Nuc. Acids Res. **11**, 2347-2365.

COSTA, R. H., DRAPER, K. G., KELLY, T. J., AND WAGNER, E. K. (1985). An unusual spliced herpes simplex virus type 1 transcript with sequence homology to Epstein-Barr virus DNA. J. Virol. **54**,317-328.

COSTANZO, F., CAMPADELLI-FIUME, G., FOA-TOMASI, L., AND CASSAI, E. (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. J. Virol. **21**, 996-1001.

CRABB, B. S., ALLEN, G. P., AND STUDDERT, M. J. (1991). Characterization of the major of glycoproteins of equine herpesviruses 4 and 1 and asinine herpesvirus 3 using monoclonal antibodies. J. Gen. Virol. **72,** 2075-2082.

CROEN, K. D., OSTROVE, J. M., DRAGOVIC, L. J., AND STRAUS, S. E. (1988). Patterns of gene expression and sites of latency in human nerve ganglia are different for varicella-zoster and herpes simplex viruses. Proc Natl. Aca. Sci. (USA). **85,** 9773-9777.

CROEN, K. D., OSTROVE, J. M., DRAGOVIC, L. J., SMIALEK J. E., AND STRAUS, S. E. (1987). Latent herpes simplex virus in human trigeminal ganglia: detection of an immediate early gene "antisense" trnascript by in situ hyridization. N. Engl. J. Med. **317,** 1427-1432

CRUTE, J. J., AND LEHMAN, I. R. (1989a). Herpes simplex-1 DNA polymerase. Identification of an intrinsic 5'-3' exonuclease with ribonucleases H. activity. J. Biol. Chem. **264**, 19266-19270.

CRUTE, J. J., TSURUMI, T., ZHU, L., WELLER, S. K., OLIVO, P. D., CHALLBERG, M. D., MOCARSKI, E. S., AND LEHMAN, I. R. (1989b). Herpes simplex virus 1 helicase-primase: a complex of three herpes-encoded gene products. Proc. Natl. Acad. Sci. USA **86,** 2186-2189.

CRUTE, J. J., MOCARSKI, E. S., AND LEHMAN, I. R. (1988). A DNA helicase induced by herpes simplex virus type 1. Nuc. Acids Res. **16,** 6585-6596.

DALES, S., AND SILVERBERG, H. (1969). Viropexis of herpes simplex virus by Hela cells. Virol. **37**, 475-480.

DAL CANTO, M. C., AND RABINOWITZ, S. G. (1981). Murine central nervous system infection by a virus temperature sensitive mutant. Amer. J. Path. **102,** 412-426.

DAMBAUGH, T., HENNESSY, K., FENNEWALD, S., AND KIEFF, E. (1986). The virus genome and its expression in latent infection. In:"The Epstein-Barr virus: Recent Advances", pp. 13-45, M. A. Eastein and B. C. Achong (eds). William Heinemann, London.

DARGAN, D. J. (1986). The structure and assembly of herpesviruses, p. 359-457. In J. R. Harris and R. W. Horned (eds), Electron microscopy of proteins, Vol. 5, Viral structure Academic Press. Inc. New York.

DARLINGTON, R. W., AND MOSS III, L. H. (1968). Herpesvirus envelopment. J. Virol. **2,** 48-55.

DAVISON, A. J. (1993). Herpesvirus genes. Reviews in medical Virology **3**, 237-244.

DAVISON, A. J., and WILKIE, N. M. (1983 a). Location and orientation of homologous sequences in the genomes of five herpesviruses. J. Gen. Virol. **64**, 1927-1942.

DAVISON, A. J., AND WILKIE, N. M. (1983 b). Inversion of the two segments of the herpes simplex virus genome in intertypic recombinants. J. Gen. Virol. **64**, 1-18.

DAVISON , A. J., AND SCOTT, J. E. (1985). DNA Sequence of the major inverted repeat in the varicella-zoster virus genome. J. Gen. Virol. **66**, 207-220.

DEAN, H. J., TERHUNE, S. S., SHIEH, M-T., AND SPEAR, P. G. (1994). Single amino acid substitutions in glycoprotein D of herpes simplex virus type 1 confer resistance to gD-mediated interference and cause cell-type-dependent alteration in infectivity. Virol. **199,** 67-80.

DEBROY, C., PEDERSON, N., AND PERSON, S. (1985). Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. Virology **145,** 36-48.

DESAI, P. J., SCHAFFER, P. A., AND MINSON, A. C. (1988). Excretion of noninfectious virus particles lacking glycoprotein H by a temperature-sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virus infectivity. J. Gen. Virol. **69**, 1147-1156.

de BRUYN KOPS, A., AND KNIPE, D. M. (1988). Formation of DNA replication structures in the herpes virus induced cells requires a viral DNA binding protein. Cell, **55.** 857-868.

DOLAN, A., ARBUCKLE, M., AND MCGEOCH, D. J. (1991). Sequence analysis of the splice junction in the transcript of herpes simplex virus type 1 gene UL15. Virus Res. **20**, 97-104.

DOLL, E. R., AND BRYANS, J. T. (1963). A planned infection program for immunizing mares against rhinopneumonitis. Cornell Vet, **53,** 249-262.

DOLL, E. R., AND BRYANS, J. H. (1962). Incubation periods for abortion in equine viral rhinopneumonitis. J. Am. Vet. Med. Assn. **141**, 351-354.

DOLL, E. R., BRYANS, J. T., MCCOLLEM, W. H., AND CROWE, M. E. W. (1957). Isolation of a filterable aget causing arteritis of horses and abortion by mares: its differentiation from the equine abortion (influenza) virus. Cornell Vet. **47,** 3-41.

DOLL, E. R., BRYANS, J. T., MCCOLLUM, W. H., AND CROWE, E W. (1956). Propagation of equine abortive virus in Syrian hamsters. Cornell Vet. **46**, 68-82.

DOMS, R.W., RUSS, G., AND YEWDELL, J. W. (1989). Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. J. Cell Biol. **109,** 61-72.

DUBUISSON, J., ISRAEL, B. A., AND LETCHWORTH III, G. J. (1992). Mechanisms of bovine herpesvirus type 1 neutralisition by monoclonal antibodies to glycoproteins gI gIII and gIV. J. Gen. Virol. **73**:2031-2039.

DWYERNIELD, L. D., MILLER, A. C. K., NEIGHBORS, B. W., DINSDALE, D., AND MALKINSON, A. M. (1996). Cytoskeletal architecture in mouse lung epithelial cells is regulated by protein kinase C-alpha and calpain II. American J. Physiology-lung cellular and molecular physiology. **270**, L526-L534.

EDINGTON, N., BRIDGES, C. G., AND HUCKLE, A. (1985). Experimental reactivation of equid herpesvirus 1 (EHV1) following the administration of corticosteroids. Equine Vet. J. **17**, 369-372.

EFSTATHIOU, S., MINSON, A. C., FIELD, H. J., ANDERSON, J. R., AND WILDY, P. (1986). Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. J. Virol. **57,** 446-455.

EHLERS, B., BUHK, H. J., AND LUDWIG, H. (1985). Analysis of bovine Cytomegalovirus genome structure: Cloning and mapping of the monomeric polyrepetitive DNA unit, and comparison of European and American strains. J. Gen. Virol. **66,** 55-68.

EISENBERG, R. J., PONCE DE LEON, M., FRIEDMAN, H. M., FRIES, L. F., FRANK, M. M., HASTINGS, J. C., AND COHEN, G. H. (1987). Complement component

C3b binds directly to purified glycoprotein C of herpes simplex virus type 1 and 2. Microbiol Pathogenesis. **3,** 423-435.

ELDER, J. H., AND ALEXANDER, S. (1982). Endo-ß-N-acetylglucosaminidase F: Endoglycosidase from *flavobacterium meningosepticum* that cleaves both high-mannose and complex glycoproteins. Proc. Natl. Aca. Sci. USA **79,** 4540-4544.

ELIAS, P., AND LEHMAN, I. R. (1988). Interaction of origin binding protein with an origin of replication of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **85,** 2959-2963.

ELLIOTT, G., AND O'HARE, P. (1995). Equine herpesvirus 1 gene 12, the functional homologue of herpes simplex virus VP16, Transactivation via octamer sequences in the equine herpesvirus IE gene promoter. Virol. **213**, 258-262.

ELTON, D. M., HALLIBURTON, I. W., KILLINGTON, R. A., MEREDITH, D. M., AND BONASS, W. A. (1991). Sequence analysis of the 4.7 Kb BamHI-EcoRI fragment of the equine-herpesvirus type 1 short unique region. Gene **101**, 203-208.

EPHRUSSI, A., CHURCH, G. M., TONEGAWA, S., AND GILBERT, W. (1985). B lineage-specific interactions of an immunoglobulin enhancer with cellular factor *in vivo*. Science **227**, 134-140.

EPSTEIN, M. A., ACHONG, B. G., AND BARR, Y. M. (1964). Virus particles in cultured lymphocytes from Burkitt's lymphoma. Lancet **1,** 702-703.

ERASMUS, B. J. (1970). Equine cytomegaloviruses. In: Pro. 2nd Int. Conf. Equine Inf. Dis. pp. 46-55. Edited by J. T. Bryans and H. Gerber, Karger, Basel.

FARRELLY, B. T. (1966). Abortion-associated with equine rihnopneumonitis viral infection in Ireland. In: proc. 1st Conf. Equine Inf. Dis. pp. 131-136. Edited by J. T. Bryans.

FELGNER, P. L., GADEK, T. R., HOLM, M., ROMAN, R., CHAN, H. W., WENZ, M., NORTHROP, J. P., RINGOLD, G. M., AND DANIELSEN, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acd. Sci. USA **84,** 7413-7417.

FENWICK, M. L., AND WALKER, M. J. (1978). Suppression of the synthesis of cellular macromolecules by herpes simplex virus. J. Gen. Virol. **41**, 37-51.

FENWICK, M. L. (1984). The effects of herpes viruses on cellular macromolecular synthesis in:"comprehensive virology" vol. 19. pp. 359-390. H. Fraenkel-Conrat and R. R. Wagner (eds) Plenum Press. N. Y.

FIELD, H. J., AND WILDY, P. (1978). The pathogenicity of thymidine kinase deficient mutants of herpes simplex virus in mice. J. Hygiene **81**, 267-277.

FLOWERS, C. C., EASTMAN, E. M., AND O'CALLAGHAN, D. J. (1991). Sequence analysis of a glycoprotein D gene homolog within the unique short segment of the EHV-1 genome. Virol. **180**, 175-184.

FORRESTER, A., FARRELL, H., WILKINSON, G., KAYE, J., DAVIS-POYNTER, N., AND MINSON, T. (1992). Construction and properties of a mutant of herpes simplex virus type with glycoprotein H coding sequence deleted. J. Virol **66,** 341-348

FRAME, M. C., PURVES, F. C., MCGEOCH, D. J., MARSDEN, H. S., AND LEADER, D. P. (1987). Identification of the herpes simplex virus protein kinase as the product of viral gene US3. J. Gen. Virol. **68,** 2699-2704.

FRANK, I., AND FRIEDMAN, H. M. (1989). A novel function of the herpes simplex virus type 1 Fc receptor: participation in bipolar bridging of antiviral IgG. J. Virol. **63,**4479-4488.

FRENKEL, N., SCHIRMER, E. C., WYATT, L. S., KATSAFANAS, G., ROFFMAN, E., DANOVICH, R. M., AND JUNE, C. H. (1990). Isolation of a new herpesvirus from human $CD4^+$ T cells. Proc. Natl. Acad. Sci. USA **87,** 748-752.

FRENKEL, N. (1981). Defective interfering herpesvirus, pp. 19-120. In: A. J. Nahmias, W. R. Dowdle, and R. F. Schinaz (eds),The human herpesvirus: an interdisciplinary perspective. Elsevier/North-Holland, Publishing Co. , New York.

FRIEDMAN, H. M., COHEN, G. H., EISENERG, R. J., SEIDEL, C. A., AND CLINES, D. B. (1984). Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. Nature 633-635.

FRIEDMAN, A., SHLOMAI, J., AND BECKER, Y. (1977). Electron microscopy of herpes simplex virus DNA molecules isolated from infected cells by centrifugation in CsCl density gradients. J. Gen. Virol. **34,** 507-522

FULLER, A. O., AND LEE, W. C. (1992). Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. J. Virol. **66**:5002-5012.

FULLER, A. O., AND SPEAR, P. G. (1987). Anti-glycoprotein D antibodies that permit adsorption but block infection of herpes simplex virus 1 prevent virion-cell fusion at the cell surface. Proc. Natl. Acad. Sci. USA **84**, 5454-5458.

FULLER, A. O., AND SPEAR, P. G. (1985). Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralizing antibodies. J. Virol. **55**, 475-482.

FURLONG, D. (1978). Direct evidence for 6-fold symmetry of the herpesvirus hexon capsomere. Proc. Natl. Acad. Sci. USA **75**, 2764-2766.

GENTRY, G. A., AND RANDALL, C. C. (1960). Studies on hepatitis in hamsters infected with equine abortion virus. III. Nuclear protein changs. A histochemical study. Am. J. Pathol. **37,** 433-441.

GENTRY, G. A., RANDALL, C. C., AND DARLINGTON, R. W. (1960). Composition of RNA and DNA of citric acid-isolated liver nuclei from hamsters infected with equine abortion virus (EAV). Virol. **11,**773-775.

GERSTER, T., AND ROEDER, R. G. (1988). A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA **85**, 6347-6351.

GIBSON, R., KORNFELD, S., AND SCHLESINGER, S. (1980). The role of oligosaccharides in glycoprotein biosynthesis. Trends in Biochemical Science **5**, 290-293.

GLASGOW, L. A. AND HABEL, K. (1962). The role of interferon in vaccinia virus infection of mouse embryo culture. J. Experimental Medicine. **115**, 503-512.

GLEESON, L. J., AND COGGINS, L. (1980). Response of pregnant mares to equine herpesvirus 1 (EHV1). Cornell Vet. **70,** 391-400.

GLEESON, L. J., AND STUDDERT, M. J. (1977). Equine herpesviruses, Experimental infection of a foal with type 2 Aust. Vet. J. **53**, 360-362.

GOOD, R. A., & CAMPBELL, B. (1948). The precipitation of latent herpes simplex encephalitis by anaphylactic shock. Proc. Soc. Exp. Biol. **68,** 82-87.

GOODRICH, L. D., RIXON, F. J., AND PARRIS, D. S. (1989). Kinetics of expression of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. J. Virol. **63,** 137-147.

GORDON, Y. J., MCKNIGHT, J. L. C., OSTROVE, J. M., ROMANOWSKI, E., AND ARAULLO-CRUZ, T. (1990). Host species and strain differences affect ability of an HSV1 ICP0 deletion mutant to establish latency and spontaneously reactivate *in vivo.* Virology **178,** 469-477.

GORDON, Y. L., JOHNSON, B., ROMANOWSKI, E., AND ARAULLO-CRUZ, T. (1988). RNA complementary to herpes simplex virus type 1 ICP0 gene demonstrated in neurons of human trigeminal ganglia. J. Virol. **62,** 1832-1835.

GOUGH, J.A., AND MURRAY, N. E. (1993). Sequence diversity among related genes for recognition of specific targets in DNA moleculars. J. Molecule. Biology **166,**1-19.

GOTTLIEB, J., MARCY, A. I., COEN, D. M., AND CHALLBERG, M. D. (1990). The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. J. Virol. **64**, 5976-5987.

GRAVELL, M. (1971). Viruses and renal carcinoma of Rana pipeins. X. comparison of herpes-type viruses associated with lucké tumour-bearing frogs. Virol. **43,** 730-733.

GRAY, W. L., BAUMANN, R. P., ROBERTSON, A. T., CAUGHMAN, G. B., O'CALLAGHAN, D. J., AND STACZEK, J. (1987 a). Regulation of equine herpesvirus type 1 gene expression: characterisation of immediate early, early and late transcription. Virol. **158**, 79-87.

GRAY, W. L., BAUMANN, R. P., ROBERTSON, A. T., O'CALLAGHAN, D. J., AND STACZEK, J. (1987 b). Characterisation and mapping of equine herpesvirus type 1 immediate early, early, and late transcripts. Virus Res. **8,** 233-244.

GRAY, W. L.,, YALAMANCHILI, R., RAENGSAKULRACH, B., BAUMANN, R., STACZEK, J., AND O'CALLAGHAN, D. J. (1989). Viral transcripts in cells infected with defective interfering particles of equine herpesvirus type 1. Virol. **172**, 1-10.

HEINE, J.W., HONESS, R. W., CASSAI, E., AD ROIZMAN, B. (1974). Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. **14**, 640-651

GRUNDY, F. J., BAUMANN, R. P., AND O'CALLAGHAN, D. J. (1989). DNA sequence and comparative analysis of the equine herpesvirus type 1 immediate early gene. Vriol. **172,** 223-236.

GUO, P., GOEBEL, S., DAVIS, S., PERKUS, M. E., LANGUET, B., DESMETTRE, P., ALLEN, G., AND PAOLETTI, E. (1989). Expression in recombinant vaccinia virus of the equine herpesvirus 1 gene encoding glycoprotein gp13 and protectoin of Immunized animals. J. Virol. **63,** 4189-4198.

HAFFEY, M. L., NOVOTNY, J., BRUCCOLERI, R. E., CARROLL, R. D., STEVENS, J. T., AND MATTHEWS, J. T. (1990). Structure- function studies of the herpes simplex virus type 1 DNA polymerase. J. Virol. **64**, 5008-5018.

HARLOW, D. L. (1988). "ANTIBODIES", A Laboratory Manual. Cold Spring Harbor Laboratory. pp. 88-90.

HARRIS, R. A., AND PRESTON, C. M. (1992). Establishment of latency *in vitro* by the herpes simplex virus type 1 mutant *in* 1814. J. Gen. Virol. **72,** 907-913.

HARRIS, E., LOEFFLER, W., SIGG, H. P., STAHELIN, H., AND TAMM, H. (1963). Die konstitution von Brefeldin A. Helv.Chim. Acta **45,** 1235-1243.

HARRIS-HAMILTON, E., AND BACHENHEIMER, S. L. (1985). Accumulation of herpes simplex virus type 1 RNAs of different kinetic classes in the cytoplasm of infected cells. J. Virol. **53**, 144-151.

HARSON, R., AND GROSE, C. (1995). Egress of Varicella-Zoster virus from the melanoma cell: a tropism for the melanocyte. J. Virol. **69**, 4994-5010.

HARTY, R. N., COLLE, C. F., GRUNDY, F. J., AND O'CALLAGHAN, D. J. (1989). Mapping the termini and intron of the spliced immediate-early transcript of equine herpesvirus 1. J. Virol. **63,** 5101-5110.

HENRY, B. E., ROBINSON, R. A., DAUEHAUER, S. A., ATHERTON, S. S., HAYWARD, G. S., ADN O'CALLAGHAN, D. J. (1981). Structure of the genome of equine herpesvirus type 1. Virology, **115,** 97-114.

HEROLD, B. C., VISALLI, R. J., SUSMARSKI, N., BRANDT, C. R., AND SPEAR, P. G. (1994). Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J. Gen. Virol. **75:** 1211-1222.

HEROLD, B. C., WUDUNN, D., SOLTYS, N., AND SPEAR, P. G. (1991). Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Viral. **65,** 1090-1098.

HIGHLANDER, S. L., CAI, W., PERSON, S., LEVINE, M., AND GLORIOSO, J. C. (1988). Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. J. Virol. **62,** 1881-1888.

HIGHLANDER, S. L., SUTHERLAND, S. L., GAGE, P. S., JOHNSON, D. C., LEVINE, M., AND GLORIOSO, J. C. (1987). Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. **61**, 3356-3364.

HILL, J. M., SEDARATI, F., JAVIER, R. T., WAGNER, E. K., AND STEVENS, J. G. (1990). Herpes simplex virus latent phase transcription facilitates *in vivo* reactivation. Virology **174,** 117-125.

HIRSCH, I., CABRAL, G., PATTERSON, M., AND BISWAL, N. (1977). Studies on the intracellular replicating DNA of herpes simplex virus type 1. Virol. **81**, 48-61.

HJORTH, R. N., BONDE, G. M., PIERZCHALA, W. A., VERNON, S. K., WIENER, F. P., LEVNER, M. H., LUBECK, M. D., AND HUNG, P. P. (1988). A new hamster model for adenoviral vaccination. Archives of Virol. **100,** 279-283.

HO, D. Y., AND MOCARSKI, E. S. (1989). Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. Proc. Natl. Acad. Sci. USA **86,** 7596-7600.

HO, M., AND ENDERS, J. F. (1959). Further studies on an inhibitor of viral activity appearing in infected cell culture and its role in chronic viral infections. Virol. **9**, 446-477.

HOLDEN, V. R., CAUGHMAN, G. B., ZHAO, Y., HARTY, R. N., AND O'CALLAGHAN, D. J. (1994). Identification and characterization of the ICP22 protein of equine herpesvirus 1. J. Virol. **68**, 4329-4340.

Huemer, H. P., Nowotny, N., Crabb, B.S., and Hubert, P.H., (1995) gp13 (EHV-gC): a complement receptor induced by equine herpesvirus. Virus Res. 37, 113-126.

HOLDEN, V. R., YALAMANDILI, R. R., HARTY, R. N., AND O'CALLAGHAN, D. J. (1992). ICP22 homology of equine herpesvirus type 1: expression from early and late promoters. J. Virol. **66**, 664-673.

HOLMES, K. V., DOLLER, E. W., AND STURMAN, L. S. (1981). Tunicamycin resistant glycosylation of a coronavirus glycoprotein: Demonstration of a novel type of viral glycoprotein. Virology **115,** 334-344.

HONESS, R. W. (1984). Herpes simplex and 'the herpes complex': diverse observations and a unifying hypothesis. J. Gen. Virol. **65**, 2077-2107.

HONESS, R. W., AND WATSON, D. H. (1977). Unity and diversity in the herpesviruses. J. Gen. Virol. **37**, 15-37.

HONESS, R. W., AND ROIZMAN, B. (1975). Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polyeptides. Proc. Natl. Acad. Sci. U.S.A. **72,** 1276-1280.

HONESS, R. W., AND ROIZMAN, B. (1974). Regulation of herpesvirus macromolecular synthesis I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14,** 8-19.

HONESS, R. W., AND ROIZMAN, B. (1973). Proteins specified by herpes simplex virus XI. Identification and relative molecuar rates of synthesis of structural and nonstructural herpes virus polypeptides in infected cells. J. Virol. **12,** 1347-1365.

HSIUNG, G. D., FISCHMAN, H. R., FONG, C. K. Y., AND GREEN, R. H. (1969). Characterization of a cytomegalo-like virus isolated from spontaneously degenerated equine kidney cell culture. Pro Soc. Exp. Biol. Med. **130**, 80-84.

HUANG, A. S., AND WAGNER, R. R. (1964). Penetration of herpes simplex virus into human epidermoid cells. Pro. Soc. Exp. Biol. **116**, 863-869.

HUANG, A. S., AND BALTIMORE, D. (1977). Defective interfering viruses, pp. 73-116. In H. Frankel-Conrat and K. R. Wagner eds. Comprehensive Virology, Vol. 10 Plenum Publishing Corp., New York.

HSIUNG, G. D., FISCHMAN, H. R. FOUNG, C. K. Y., AND GREEN, R. H. (1969).

Characterization a cytomegalo-like virus isolated from spontaneously degenerated equine kidney cell culture. Proc. Soc. Exp. Biol. Medi. **130,**80-84.

HUMMELLER, K., TOMASINI N., AND ZAJAC, B. (1969). Early events in herpes simplex virus infection: A radioautographic study. J. Virol. **4.** 67-74.

HURD, J., AND ROBINSON, T. W. E. (1977). Herpes simplex virus: Aspects of reactivation in a mouse model. J. Antimicrob. Chemother. **3,** 99-106.

HUTCHINSON, L., BROWNE, H., WARGENT, V., DAVIS-POYNTER, N., PRIMORAC, S., GOLDSMITH, K., MINSON, A. C., AND JOHNSON, D. C. (1992 a). A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. **66,** 2240-2250.

HUTCHINSON, L., GOLDSMITH, K., SNODDY, D., GHOSH, H., GRANAM, F. L., AND JOHNSON, D. C. (1992 b). Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. **66**, 5603-5609.

INGEMARSON, R., AND LANKINEN, H. (1987). The herpes simplex virus type 1 ribonucleotide reductase is a tight complex of the type alpha2beta2 composed of 40K and 41K proteins, of which the latter shows multiple forms due to proteolysis. Virol. **156,** 417-422.

IHARA, S., FELDMAN, L., WATANABE, S., AND BEN-PORAT, T. (1983). Characterisation of the immediate early functions of pseudorabies virus. Virol. **131**, 437-454.

JACKSON, T. A., OSBURN, B. I., CORDY, D. R., AND KENDRICK, J. W. (1977). Equine herpesvirus 1 infection of horses: studies on the experimentally induced neurologic disease. Am. J. Vet. Res. **38**, 709-719.

JACKSON, T., AND KENDRICK, J. W. (1971). Paralysis of horses associated with equine herpesvirus 1 infection. J. Am. Vet. Med. Assn. **158,** 1351-1357.

JACOB, R. J., MORSE, L. S., AND ROIZMAN, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatamers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J. Virol. **29,** 448-457.

JACOB, R. J., AND ROIZMAN, B. (1977). Anatomy of herpes simplex virus DNA VIII. Properties of the replicating DNA. J. Virol. **23**, 394-411.

JAMIESON, A. T., GENTRY, G. A., AND SU BAK-SHARPE, J. H. (1974). Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J. Gen. Virol. **24,** 465-480.

JAVIER, R. T., STEVENS, J. G., DISSETTE, V. B., AND WAGNER, E. K. (1988). A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology **166,** 254-257.

JEAN, J. H., AND BEN-PORAT, T. (1976). Appearance *in vivo* of single-strand complementary ends on herpesvirus DNA, Proc. Natl. Acad. Sci. USA **73**, 2674-2678.

JONES, P. C., AND ROIZMAN, B. (1979). Regulation of herpes virus macromolecular synthesis VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. J. Virol. **31**,299-314.

JOHNSON, P. A., BEST, M. G., FRIEDMANN, T., AND PARRIS, D. S. (1991). Isolation of a herpes simplex virus type 1 mutant deleted for the essential UL42 gene and charaterization of its null phenotype. J. Virol. **65**, 700-710.

JOHNSON, D. C., BURKE, R. L., AND GREGORY, T. (1990). Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. J. Virol. **64**:2569-2576.

JOHNSON, R. M., AND SPEAR, P. G. (1989). Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. J Virol **63,** 819-827.

JOHNSON, D. C., AND LIGAS, M. W. (1988a). Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. **62**:4605-4612.

JOHNSON, D. C., FRAME, M. C., LIGAS, M. W., CROSS, A. M., AND STOW, N. D. (1988b). Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins gE and gI. J. Virol. **62,** 1347-1354.

JOHNSON, P. A., MACLEAN, C. A., MARSDEN, H. S., DALZIEL, R. G., AND EVERETT, R. D. (1986). The product of the gene Us11 of herpes simplex virus type 1 is expressed as a true late gene. J. Gen. Virol. **67,** 871-883.

JOHNSON, D. C., WITTELS, M., AND SPEAR, P. G. (1984). Binding to cells of virosomes containing herpes simplex virus type 1 glycoproteins and evidence for fusion. J. Virol. **52**:238-247.

JOHNSON, D. C., AND SPEAR, P. G. (1983). O-Linked oligosaccharides are attached to herpes simplex virus glycoproteins in the Golgi apparatus. Cell **32,** 987-997.

JOHNSON, D. C., AND SPEAR, P. G. (1982). Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. **43,** 1102-1112.

JöNS , A., GRANZOW, H., KUCHLING, R., AND METTENLEITER, T. C. (1996). The UL49.5 gene of pseudorabies Vvirus codes for an O-glycosylated structural protein of the vrial envelope. J. Virol. **70,** 1237-1241.

KARGER, A., AND METTENLEITER, T. C. (1993). Glycoprotein gIII and gp50 play dominant roles in the biphasic attachment of pseudorabies virus. Virology, **194,** 654-664.

KATZ, J. P., BODIN, E. T., AND COEN, D. M. (1990). Quantitative polymerase chain reaction analysis of herpes simplex virus DNA in ganglia of mice infected with replication-incompetent mutants. J. Virol. **64,** 4288-4295.

KAYE, J. F., GOMPELS, U. A., AND MINSON, A. C. (1992). Glycoprotein H of human cytomegolovirus (HCMV) forms a stable complex with the HCMV UL115 gene product. J. Gen. Virol. **73,** 2693-2698.

KEMP, M. C., PERDUE, M. L., ROGERS, H. W., O'CALLAGHAN, D. J., AND RANDALL, C. C. (1974). Structural polypeptides of the hamster strain of equine herpes virus type 1: products associated with purification. Virol. **61,** 361-375.

KIM, S. K., SMITH, R. H., AND O'CALLAGHAN, D. J. (1995). Characterization of DNA binding properties of the immediate early gene product of equine herpesvirus type 1. Virol. **213,** 46-56.

KIMMAN, T. G., POL, J. M., de WIND, N., OEI-LIE, N., BERNS, A. J., AND GIELKENS, A. L. (1992). Role of different genes in the virulence and pathogenesis of Aujeszky's disease virus. Vet. Microbiol. **33**, 45-52.

KING, A. M., STOTT, E. J., LANGER, S. J., YOUNG, K. Y., BALL, L. A., AND WERTZ, G. W. (1987). Recombinant vaccinia viruses carrying the N gene of human respiratory syncytial virus: studies of gene expression in cell culture and immune response in mice. J. Virol. **61,** 2885-2890.

KLUPP, B. G., BAUMEISTER, J., KARGER, A., VISSER, N., AND METTENLEITER, T. C. (1994). Identification and characterisation of a novel structural glycoprotein in pseudorabies virus, gL. J. Virol. **68,** 3868-3878.

KNOPT, K. W. (1979). Properties of herpes simplex virus DNA polymerase and characterization of its associated exonuclease activity. European J. Biochem., 98, 231-244.

KRISTENSSON, K. (1988) Retrograde transport of macromolecules in axons. Annu. Rev. Pharmacol. Toxicol. **18,** 97-110.

KUHN, J. E., KRAMER, M. D., WILLENBACHER,W., WIELAND, U., LORENTZEN, E. U., AND BRAUN, R. M. (1990). Identification of herpes simplex virus type 1 glycoproteins interacting with cell surface. J. Virol. **64**:2491-2497.

KURACHI, R., DAIKOKU, T., TSURUMI, K., MAENO, K., NISHIYAMA, Y., AND KURATA, T. (1993). The pathogenicity of a Us3 protein kinase-deficient mutant of herpes simplex virus type 2 in mice. Arch. Virol. **133**, 259-273.

KWONG, A. D., AND FRENKEL, N. (1989). The herpes simplex virion host shutoff function. J. Virol. **63,** 4834-4839.

KWONG, A. D., KRUPER, J. A., AND FRENKEL, N. (1988). Herpes simplex virion host shutoff function. J. Virol. **63,** 912-921.

KYTE, J., AND DOOLITTLE, R. F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**, 105-132.

LAWRENCE, W. C. (1971). Nucleic acid and protein synthesis in KB cells infected with equine abortion virus (equine herpes virus type 1). Am. J. Vet. Res. **32,** 41-49.

LEAVITT, R., SCHLESINGER, S., AND KORAFELD, S. (1971). Tunicamycin inhibits glycoprotein and multiplication of sindbis and vesicular stomatitis virus. J. Virol. **21,** 375-385.

LEE, W. C., AND FULLER, A. O. (1993). Herpes simplex virus type 1 and pseudorabies virus bind to a common saturable receptor on Vero cells that is not heparan sulfate. J. Virol. **67**:5088-5097.

LEIB, D. A., COEN, D. M., BOGARD, C. L., HICKS, K. A., YAGER, D. R., KNIPE, D. M., TYLER, K. L., AND SCHAFFER, P. A. (1989). Immediate early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J. Virol. **63,** 759-768.

LEUNG-TACK, P., AUDONNET, J. C., AND RIVIERE, M. (1994). The complete DNA sequence and the genome organization of the short unique region (US) of the bovine herpesvirus type 1 (ST strain). Virol. **199**, 409-421.

LEVY, Y., FERRO. F., GREENSPAN. D., LENNETTE, E. T. (1990). Frequent isolation of HHV-6 from saliva and high seroprevalence of the viruse in the population. Lancet **335,** 1047-1050.

LEWIS, J. B., THOMPSON, Y. G., AND CAUGHMAN, G. B. (1993). Transcriptional control of the equine herpesvirus 1 immediate early gene. Virol. **197**, 788-792.

LI, S. C., AND LI, Y. T. (1970). Study on the glycosidases of Jack Bean Meal. III. Crystallisation and properties of ß-N-acetylhexosaminidase. J. Biol. Chem. **245,** 5153-5160.

LIANG, X. P., BABIUK, L .A., den HURK, S. V. D. L., FITZPATRICK, D. R., AND ZAMB, T. J. (1991). Bovine herpesviurs 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII and gIV. J. Virol. **65**: 1124-1132.

LIGAS, M. W., AND JOHNSON, D. C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by ß-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62,** 1486-1494.

LIPPINCOTT-SCHWARTZ, J., YUAN, L. C., BONIFACINO, J. S., AND KLAUSNER, R. D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with Brefeldin A: evidence for membrane cycling from Golgi to ER. Cell **56**, 801-813.

LITTLE, S. P., AND SCHAFFER, P. A. (1981). Expression of the syncytial (syn) phenotype of HSV1 strain KOS: genetic and phenotypic studies of mutants in two syn loci. Virology **112,** 686-702.

LITTLE, P. B., AND THORSEN, J. (1976). Disseminated necrotizing myloencephalitis: A herpes-associated neurological disease of horses. Vet. Pathol. **13**, 161-171.

LIU, D. X., GOMPELS, U. A. NICHOLAS, J., AND LELLIOTT, C. (1993). Identification and expression of the human herpesvirus 6 glycoprotein H and interaction with an accessory 40K glycoprotein. J. Gen. Virol. **74,** 1847-1857.

LONGNECKER, R., AND ROIZMAN, B. (1987). Clustering of genes dispensible for growth in culture in the S Component of the HSV1 genome. Science, **236,** 573-576.

LONSDALE. D. M. (1979). A rapid technique for distinguishing herpes simplex virus type 1 from type 2 by restriction technology. Lancet **i**, 849-852.

LUDWIG, H., BISWAL, N., BRYANS, J. T., AND MCCOMBS, R. M. (1971). Some properties of the DNA from a new equine herpesvirus. Virology **45,** 534-537.

MCCLAIN, D. S., AND FULLER, A. O. (1994). Cell-specific kinetics and efficiency of herpes simplex virus type 1 entry are determined by two distinct phases of attachment. Virology **198:** 690-720.

MCCOMBS, R. M., BRANSCHWIG, J. P., MIRKOVIC, R., AND BENYESH-MELNICK, M. (1971). Electron Microscopic charaterization of a herpeslike virus isolated from tree shrews. Virology, **45,** 816-820.

MCCRACKEN, R. M., AND CLARKE, T. K. (1971). A thin section study of the morphogenesis of Auijesky's disease virus in synchronously infected cell culture. Archiv. Ges. Virusforsch. **34,** 189-201.

MCGEOCH, D. J., BARNETT, B. C., AND MACLEAN, C. A. (1993). Emerging functions of alphaherpesvirus genes. Seminars in Virology **4** , 125-134.

MCGEOCH, D. J., DALRYMPLE, M. A., DOLAN, A., MCNAB, D., PERRY, L. J., TAYLOR, P., AND CHALLBERG, M. D. (1988a). Structures of herpes simplex virus type 1 required for replication of virus DNA. J. Virol. **62,** 444-453.

MCGEOCH, D. J., DALRYMPLE, M. A., DAVISON, A. J., DOLAN, A., FRAME, M. C., MCNAB, D., PERRY, L. J., SCOTT, J. E., AND TAYLOR, P. (1988b). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69,** 1531-1574.

MCGEOCH, D. J., MOSS, H. W. M., MCNAB, D., AND FRAME, M. C. (1987). DNA sequence and genetic content of the HindIII L region in the short unique component of the herpes simplex type 2 genome: Identification of the gene encoding glycoprotein G and evolutionary comparisons. J. Gen. Virol. **68**, 19-38.

MCGEOCH, D. J., AND DAVISON, A. J. (1986 a). Alpha herpes viruses possess a gene homologous to the protein kinase gene family of eukaryotes and retroviruses. Nucleic Acids Res. **14,** 1765-1777.

MCGEOCH, D. J., DOLAN, A., DONALD, S., AND BRAUER, D. H. K. (1986 b). Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucl. Acids. Res. **14,** 1727-1745.

MCGEOCH, D. J. (1985). On the predictive recognition of signal peptide sequences. Virus Res. **3,** 271-286.

MCGEOCH, D. J., DOLAN, A., DONALD, S., AND RIXON, F. J. (1985b). Sequence determination and genetic content of the short unique region in the genome of herpe simplex virus type 1. J. Mol. Biol. **181**, 1-13.

MCGOWAN, E. M., HAYDEN, M. B., EDWARDS, S. J., PYE, D., LOVE, D. N., AND WHALLEY, J. M. (1994). Expression and characterisation of equine herpesvirus 1 glycoprotein H using a recombinant baculovirus. Arch. Virol. **137**, 389-395.

MCKNIGHT, J. L. C., KRISTIE, T. M., AND ROIZMAN, B. (1987). Binding of the vivion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its site requires cellular proteins. Proc. Natl, Acad. Sci. USA **84,** 7061-7065.

MCKNIGHT, S. L., AND KINGSBURY, R. (1982). Transcriptional control signals of a eukaruotic protein coding gene. Science **217,** 316-324.

MCKNIGHT, S. L. (1980). The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res. **8**, 5949-5960.

MCLAUCHLAN, J., AND RIXON, F. J. (1992). Characterization of envelope tegument structures (L particles) produced by alphaherpesviruses:integrety of the tegument does not depend on the presence of capsid or envelope. J. Gen. Virol. **73,** 269-276.

MCLENNAN, J. L., AND DARBY, G. (1980). Herpes simplex virus latency: the cellular location of virus in dorsal root ganglia and the fate of the infected cell following virus activation. J. Gen. Virol. **51,** 233-243.

MCNEARNEY, T. A., ODELL, C., HOLERS, V. M., SPEAR, P. G., AND ATKINSON, J. P. (1987). Herpes simplex virus glycoproteins gC-1 and gC-2 bind the third component of complement and provide protection against complement-mediated neutralization of viral infectivity. J. Exp. Med. **166**, 1525-1535.

MACKEM, S., AND ROIZMAN, B. (1982 a). Differentiation between alpha-promoter and regulatory regions of herpes simplex virus 1: the functional domains and sequence of a movable alpha regulator. Proc. Natl. Sci. USA **79**, 4917-4921.

MACKEM, S., AND ROIZMAN, B. (1982 b). Structural features of the herpes simplex virus α gene 4, 0 and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44,** 939-949.

MACKEM, S., AND ROIZMAN, B. (1982 c). Regulation of the alpha genes of herpes simplex virus: The alpha 27 gene promoter thymidine kinase chimera is positively regulated in converted L cells. J. Virol. **43**, 1015-1-23.

MACKEM, S., AND ROIZMAN, B. (1981). Regulation of herpes virus macromolecular synthesis: Temporal order of transcription of alpha-genes is not dependent on the stringency of inhibition of protein synthesis. J. Virol. **40**, 319-322.

MACKEM, S., AND ROIZMAN, B. (1980). Regulation of herpes virus macromolecular synthesis: Transcription-initiaton sites and domains of alpha genes. Pro. Natl. Acad. Sci. USA. **77**, 7122-7126.

MACLEAN, C. A., DOLAN, A., JAMIESON, F. E., AND MCGEOCH, D. J. (1992). The myristylated virion proteins of herpes simplex virus type 1: Investigation of their role in the virus life cycle. J. Gen. Virol. **73,** 539-547.

MACLEAN, C. A., EFSTATHIOU, S., ELLIOTT, M. L., JAMIESON, F. E., AND MCGEOCH, D. J. (1991). Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. J Gen Virol **72,**897-906.

MACLEAN, C. A., CLARK, B., AND MCGEOCH, D. J. (1989). Gene UL11 of herpes simplex virus type 1 encodes a virion protein which is myristylated. J. Gen. Virol. **70,** 3147-3157.

MACPHERSON, I., AND STOKER, M. (1962). Polyoma transformation of hamster cell clones: an investigation of genetic factors affecting cell competence. Virol. **16,** 147-151.

MAHONEY, W. C., AND DUKSIN, D. (1979). Biological activities of the two major components of tunicamycin. J. Biol. Chem. **254**, 6572-6576.

MALEY, F., TRIMBLE, R. B., TARENTINO, A. L., AND PLUMMER, T. H. Jr. (1989). Characterisation of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. Anal. Biochem. **180,** 195-204.

MANIATIS, T., FRITSHCH, E. F., AND SAMBROOK, J. (1982). "Molecular Cloning; A Laboratory Manual". N. Y. : Cold Spring Harbour Laboratory Publication.

MANSERVIGI, R., SPEAR, P. G., AND BUCHAN, A. (1977). Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA **74,** 3913-3917.

MARCHETTI, M. E., SMITH, C. E., AND SCHAFFER, P. A. (1988). A temperature-sensitive mutation in a herpes simplex virus type 1 gene required for viral DNA synthesis maps to coordinates 0.609 through 0.614 in UL. J. Virol. **62,** 715-721.

MARCY, A. I., OLIVO, P. D., CHALLBERG, M. D., AND COEN, D. M. (1990). Enzymatic activities of overexpressed herpes simplex virus DNA polymerase purified from recombinant baculovirus infected insect cells. Nucl. Acids Res. **18**, 1207-1215.

MARSDEN, H. S., CAMPBELL, M. E. M., HAARR, L., FRAME, M. C., PARRIS, D. S., MURPHY, M., HOPE, R. G., MULLER, M. T., AND PRESTON, C. M. (1987). The 65,000 Mr DNA-binding and virion trans-inducing proteins of herpes simplex virus type 1. J. Virol. **61**, 2428-2437.

MARTIN, R. G., DAWSON, C. R., JONES, P., TOGNI, B., LYONS, C., AND OH, J. O. (1977). Herpes virus in sensory and autonomic ganglia after eye infection. Arch. Ophthalmol. **95,** 2053-2056.

MATTHEWS, R. E. F. (1982). Classification and nomenclature of viruses. Intervirology **17,** 1-199.

MATTHEWS, R. E. F. (1979). Classification and nomenclature of viruses. Intervirology **12,**165-169.

MATUMOTO, M., ISHIZAKI, R., AND SHIMIZU, T. (1965). Serological survey of equine rhinopneumonitis virus infection among horses in various countries. Arch Ges. Virusforsch **15,** 609-623.

MAYR, A., PETTE, J., PETZOLDT, K., AND WAGENER, K. (1968). Untersuchungen zur Entwicklung eines lebendimpfstoffes genen die rhinopneumonitis (studenabort) des pferdes. J. Vet. Med. B **15**. 406-418.

MAYR, A., BOHM, H., BRILL, J., AND WOYCIERCHOWSKA, S. (1965). Charakterisierung eines studenabortvirus aus polen und vergleich mit bekannten rhinopneumonitis- virus-stammen des pferdes. Arch. ges, Virusforsch **17,** 216-230.

MEIGNEIR, B., LONGNECKER, R., MAVROMARA-NAZOS, P., SEARS, A. E., AND ROIZMAN, B. (1988). Virulence and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1. Virology **162,** 251-254.

MEREDITH, D. M., STOKES, J-M., WHITTAKER, G. R., HALLIBURTON, I. W., SNOWDEN, B. W., AND KILLINGTON, R. A. (1989). Identification of the gB homologues of equine herpesvirus types 1 and 4 as disulphide-linked heterodimers and their characterization using monoclonal antibodies. J.Gen. Virol. **70**, 1161-1172.

METTENLEITER, T. C., ZSAK, L., ZUCKERMANN, F., SUGG, N., KERN, H., AND BEN-PORAT, T. (1990). Interaction of glycoprotein gIII with a cellular herparinlike substance mediates adsorption of pseudorabies virus. J. Virol. **64:**278-286.

KLYMKOWSKY, M.W., AND KARNOVSKY, A. (1994), Morphogenesis and the cytoskeleton: Studies of the xenopus embryo. Developmental Bio. **165**, 372-384.

move after KING, A. M., ...

MINSON, A. C., HODGMAN, T. C., DIGARD, P., HANCOCK, D. C., BELL, S. E., AND BUCKMASTER, E. A. (1986). An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralisation. J. Gen. Virol. **67,** 1001-1013.

MISRA, V., AND BLEWETT, E. L. (1991). Construction of herpes simplex viruses that are pseudodiploid for the glycoprotein B gene: a strategy for studying the function of an essential gene. J. Gen.Virol. **72**, 385-392.

MISUMI, Y., MIKI, K., TAKULSUKI, A., TAMUAR, G., AND IKEHARA, Y. (1986). Novel blockade by brefeldin A intracellular transport of secretory proteins in cultured hepatocytes. J. Biol. Chem. **261,** 11398-11403.

MORGAN, C., ROSE, H. M., AND MEDNIS, B. (1968). Electron microscopy of herpes simplex virus. I. Entry J. Virology **2,** 507-516.

MORGAN, C., ELLISON, S. A., ROSE, H. M., AND MOORE, D. H. (1954). Structure and development of viruses as observed in the electron microscope. I. Herpes simplex virus. J. Exptl. Med. **100**,195-202.

MULLANEY, J., MOSS, H. W. M., AND MCGEOCH, D. J. (1989). Gene UL2 of herpes simplex virus type 1 encodes a uracil-DNA glycosylase. J. Gen. Virol. **70,** 449-454.

MUMFORD, J. A. (1985). Equine herpesvirus 1 (EHV-1) latency: More questions than answers. Equine Vet. J. **17** 340-342.

MUMFORD, J. A., AND ROSSDALE, P. D. (1980). Virus and its relationship to the "poor performance" syndrome. Equine Vet. J. **12,** 3-9.

MUMFORD, J. A., AND THOMPSON, S. R. (1978). Serological methods for identification of slowly-growing herpesviruses isolated from the respiratory tract of horses. In: Proc. 4th Int. Equine Inf. Dis. pp. 49-52. Edt. by J.T. Bryans and H. Gerber. Vet. Publ. Inc. U.S.A.

MURCHIE, M. J., AND MCGEOCH, D. J. (1982). DNA sequence analysis of an immediate-early gene region of the herpes simplex virus type 1 genome (map coordinates 0.950-0.978). J. Gen. Virol. **62**, 1-15.

NAGESHA, H. S., CRABB, B. S., AND STUDDERT, M. J. (1993). Analysis of the nucleotide sequence of five genes at the left end of the unique short region of the equine herpesvirus 4 genome. Archives of Virol. **128,** 143-154.

NASH, A. A., LEUNG, K., AND WILDY, P. (1985). The T-cell mediated response of mice to herpes simplex virus. In the herpesviruses, Vol. 4, pp. 87-102. Edited by B. Roizman and L. Lopez. New York: Plenum Press.

NAVARRO, D., PAZ, P., AND PEREIRA, L. (1992). Domains of herpes simplex virus 1 glycoprotein B that function in virus penetration, cell to cell spread and cell fusion. Virol. **186**, 99-112.

NEWCOMB, W. W., BROWN, J. C., BOOY, F. P., ,AND STEVEN, A. C. (1989). Nucleocapsid mass and capsomer protein stoichiometry in equine herpesvirus 1: Scanning transmission electron microscopic. study. J. Virol. **63,** 3777-3783.

NIEMANN, H., GEYER, R., KLENK, H.-D., LINDER, D., STIRM, S., AND WIRTH, M. (1983). The carbohydrates of mouse hepatitis virus (MHV) A59: Structures of the O-glycosidically linked oligosaccharides of glycoprotein E1. EMBO J. **3,** 665-670.

NIEMANN, H., BOSCHEK, B., EVANS, D., ROSING, M., TAMURA, T., AND KLENK, H.-D. (1982). Post-translational glycosylation of coronavirus glycoprotein E1: inhibition by monensin. EMBO J. **1,**1499-1504.

NISHIYAMA, Y., YAMADA, Y., KURACHI,R., AND DAIKOKU, T. (1992). Construction of a Us3 lacZ insertion mutant of herpes simplex virus type 2 and characterization of its phenotype *in vitro and in vivo.* Virol. **190,** 256-268.

NISHIOKA, Y., AND SILVERSTEIN, S. (1977). Degradation of cellular mRNA during infection with herpes simplex virus. Proc. Natl. Aca. Sci. (U.S.A.) **74,** 2370-2374.

NOBLE, A. G., LEE, G. T. Y., SPRAGUE, R., PARISH, M. L., AND SPEAR, P. G. (1983). Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 2. Virology **129,** 218-224.

O'CALLAGHAN, D. J., COLLE III, C. F., FLOWERS, C. C., SMITH, R. H., BENOIT, J. N., AND BIGGER, C. A. (1994). Identification and initial charactersization of the IR6 protein of equine herpesvirus 1. J. Virol. **68**, 5351-5364.

O'CALLAGHAN, D. J., GENTRY, G. A., AND RANDALL, C. C. (1983). The equine herpesviurses. In 'The herpesviruses", Vol. **2**, pp. 215-318. Edited by B. Roizman. PLENUM PRESS, N.Y.

O'CALLAGHAN, D. J., ALLEN, G. P., AND RANDALL, C. C. (1978). Structures and replication of the equine herpesviruses. In: Proc. 4th Int. Conf. Equine Inf. Dis. pp, 1-32. edt. by J.T. Bryans and H. Gerber. Vet. Publ. Inc. Princeton, New Jersey, U.S.A.

O'CALLAGHAN, D. J., AND RANDALL, C. C. (1976). Molecular anatomy of herpesviruses: Recent studies. Prog. Med. Virol. **22,** 152-210.

O'CALLAGHAN, D. J., RANDALL, C. C., AND GENTRY G. A. (1972). Herpesvirus replication in vivo. Virol. **49**, 784-793.

O'CALLAGHAN, D. J., HYDE, J. M., GENTRY, G. A., AND RANDALL, C. C. (1968 a). Kinetics of viral deoxyribonucleic acid protein, and infectious particles reduction and alterations in host macromolecular synthesis in equine abortion (herpes), virus-infected cells. J. Virol. **2,** 793-804.

O'CALLAGHAN, D. J., CHEEVERS, W. P., GENTRY, G. A., AND RANDALL, C. C. (1968 b). Kinetics of cellular and viral DNA synthesis in equine abortion (herpes) virus infection of L-M cells. Virol. **36**, 104-114.

OCHES, S. (1974) Systems of material transport in nerve fibres (axonal transport) related to nerve function and trophic control. Ann. N. Y. Acad. Sci. **288,** 202-223.

O'HARE, P., AND GODING, C. R. (1988). Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivition. Cell **52**, 435-445.

O'HARE, P., AND HAYWARD, G. S. (1985). Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early expression in a pathway involving positive and negative feedback regulation. J. Virol. **56,** 723-733.

OLIVO, P. D., NELSON, N. J., AND CHALLBERG, M. D. (1988). Herpes simplex virus DNA replication: The UL9 gene encodes an origin-binding protein. Proc. Natl. Acad Sci. USA **85**, 5414-5418.

OLOFSSON, S., BLOMBERG, J., AND LYCKE, E. (1981). O-glycosidic carbohydrate-peptide linkages of herpes simplex virus glycoproteins. Arch. Vriol. **70,** 321-329.

ONIONS, D. (1991). Equine herpesvirus: New approaches to an old problem. Eq. Vet. J. **23,** 6-7.

OPENSHAW, H., SHAVRINA ASHER, L.V. S., WOHLENBERG, C., SEKIZAWA, T., AND NOTKINS, A. L. (1979). Acute and latent infection in sensory ganglia with herpes simplex virus: Immune control and virus reactivation. J. Gen. Virol. **44,** 205-215.

OSTERRIEDER, N., HOLDEN, V. R., BTANDMüLLER, C., NEUBAUER, A., KAADEN, O., AND O'CALLAGHAN, D. J. (1996a). The equine herpesvirus 1 IR6 protein is nonessential for virus growth *in vitro* and modified by serial virus passage in cell culture. Virol. **217,** 442-451.

OSTERRIEDER, N., NEUBAUER, A., BRANDMüLLER, E. B., BRAUN, B., KAADEN, O., AND BAINES, J. D. (1996b). The equine herpesvirus glycoprotein gp21/22a, the herpes simplex virus type 1 gM homolog, is involved in virus penetration and cell to cell spread of virions. J. Virol. **70**, 4110-4115.

OSTERRIEDER, N., WAGNER, R., BRANDMüLLER, C., SCHMIDT, P., WOLF, H., AND KAADEN, O. (1995). Protection against EHV1 challenge infection in murine model after vaccination with various formulations of recombinant glycoprotein gp14 (gB). Virology, **208,** 500-510.

OSTERRIEDER, N., HüBERT, P. H., BRANDMüLLER, C., AND KAADEN, O.-R. (1994 a). A Touchdown multiplex PCR for the differentiation of equine herpesvirus type 1 (EHV-1) field strains from the modified live vaccine strain RacH. J. Virol. Methods *50* 129-136.

OSTERRIEDER, N., HüBERT, P. H., BIRKENMAIER, S., MEYER, H., AND KAADEN, O. R. (1994 b). Genome alterations in the modified live EHV-1 vaccine strain RacH. Abstracts of the 19th International Herpesvirus Workshop, Vancouver, Canada, 1994.

PACHCIARZ, J. A., AND BRYANS, J. T. In: Proc. Fourth Internat. Conf. Equine Infect. Dis., LYON, 1976 (Ed. by T. B. Bryans, and H. Gerber) Vet. Publ. Inc., Princeton, N. J. 1978, pp. 115-127.

PAPP-VID, G., AND DERBYSHIRE, J. B. (1979). The virus neutralizing activities of antibodies specific to the envelope and nucleocapsid of equine herpesvirus type 1. Canad. J. Comp. Med. **43,**231-233.

PATEL. J. R., AND EDINGTON, N. (1983). The pathogenicity in mice of respiratory, abortion and paresis isolates of equine herpesvirus-1. Vet. Microb. **8**, 301-305.

PATEL. J. R., EDINGTON, N., AND MUMFORD, J. A. (1982). Variation in cellular tropism between isolates of equine herpesvirus-1 in foals. Achives Virol. **74,** 41-51.

PATERSON, T., AND EVERETT, R. D. (1988). Mutational dissection of the HSV-1-immediate-early protein Vmw 175 involved in transcriptional transactivation and repression. Virol. **166**, 186-196.

PEETERS, B., DE WIND, N., BROER, R., GIELKENS, A., AND MOORMANN, R. (1992a). Glycoprotein H of pseudorabies virus is essential for entry and cell-to-cell spread of the virus. J. Virol **66,** 3888-3892.

PEETERS, B., DE WIND, N., HOOISMA, M., WAGENAAR, F., GIELKENS, A., AND MOORMANN, R. (1992b). Pseudorabies virus envelope glycoprotein gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. J Virol **66**, 894-905.

PERDUE, M. L., COHEN, J. C., KEMP, M. C., RANDALL, C. C., AND O'CALLAGHAN, D. J. (1975). Characterization of three species of nucleocapsides of equine herpesvirus type 1 (EHV1). Virology. **64,** 187-204.

PERDUE, M. L., KEMP, M. C., RANDALL, C. C., AND O'CALLAGHAN, D. J. (1974). Studies of the molecular anatomy of L-M cell strain of equine herpes type 1: proteins of the nucleocapsid and intact virion . Virol. **59,** 201-216.

PETROVSKIS, E. A., MEYER, A. L., AND POST, L. E. (1988). Reduced yield of infectious pseudorabies virus and herpes simplex virus from cell lines producing viral glycoprotein gp50. J Virol **62**, 2196-2199.

PILLING, A., DAVISON, A. J., TELFORD, E. A. R., AND MEREDITH, D.M. (1994). The equine herpesvirus type 1 glycoprotein homologous to herpes simplex virus type 1 glycoprotein M is a major constituent of the virus particle. J. Gen. Virol. **75,**439-442.

PIZER, L. I., COHEN, G. H., AND EIENBERG, R. J. (1980). Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. J. Virol. **34**, 142-153.

PLATT, H. (1973). Aetiological aspects of abortion in the thoroughbred mare. J. Comp. Pathol. Ther. **83,** 199-205.

PLUMMER, G., GOODHEART, C. R., AND STUDDERT, M. J. (1973). Equine herpesviruses: antigenic relationships and deoxyribonucleic acid densities. Infect. Immun. **8**, 621-627.

PLUMMER, G., BOWLING, C. P., AND GOODHEART, C. R. (1969). Comparison of four horse herpesviruses. J. Virol. **4,** 738-741.

PLUMMER, G., AND WATERSON, A. P. (1963). Equine herpesviruses. Virology **19**, 412-416.

POGUE-GEILE, K. L., AND SPEAR, P. G. (1987). The single base pair substitution responsible for the Syncytial phenotype of herpes simplex virus type 1, strain MP. Virology **157,**67-74.

POLIQUIN, L., LEVINE, G., AND SHORE, G. C. (1985). Involvement of the Golgi apparatus and a restructured nuclear envelope during biogenesis and transport of herpes simplex virus glycoproteins. J. Histochem. Cytohem. **33**, 875-883.

POST, L. E., MACKEM, S., AND ROIZMAN, B. (1981). Regulation of the alpha-genes of herpes simplex virus: expression of chimerical genes produced by fusion of thymidine kinase with alpha-gene promoters. Cell, **24,** 555-565.

POWELL, D. J. (1979). Structure and expression of the pseudorabies virus genome, Ph.D. thesis University of Glasgow.

PRESSMAN, B. C. (1976). Biological applications of ionophores. Annu. Rev. Biochem. **45,** 501-530.

PRESTON, V. G. (1990). Hepers simplex virus activates expression of a cellular gene by specific binding to the cell surface. Virology **176,** 474-482.

PRESTON, C. M., FRAME, M. C., AND CAMPBELL, M. E. M. (1988). A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell **52,** 425-434.

PRESTON, V. G., AND FISHER, F. B. (1984). Identification of the herpes simplex virus type 1 gene encoding the dUTPase. Virol. **138,** 58-68.

PRICE, R. W., AND SCHMITZ, J. (1978). Reactivation of latent herpes simplex virus infection of the autonomic nervous system by post ganglionic neurectomy. Infect. Immun. **19,** 523-532.

PRICE, R. W., KATZ, B. J., AND NOTKINS, A. L. (1975). Latent infection of the peripheral ANS with herpes simplex virus. Nature **257,** 686-688.

PRICKETT, M. E. (1970). The pathology of disease caused by equine herpesvirus 1. In: proc. 2nd Int. Conf. Eq. Inf. Dis. pp,24-33 (Eds. J. T. Bryans and H. Gerber), Karger, Basel.

PRUIJIN, G. J. M., VAN DRIEL, W., VAN DER., AND VLIET, P. C. (1986). Nuclear factor III, a novel sequence-specific DNA-binding protein from Hela cells stimulating adenovirus DNA replication. Nature **332,** 565-569.

PUREWAL, A. S., ALLSOPP, R., RIGGIO, M., TELFORD, E. A. P., AZAM, S., DAVISON, A. J., AND EDINGTON, N. (1994). Equid herpesviruses 1 and 4 encode functional homologs of the herpes simplex virus type 1 virion transactivator protein, VP16. Virology **198,** 385-389.

QUINLAN, M. P., CHEN, L. B., AND KNIPE, D. M. (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell **36**,857-868.

QUINN, J. P., AND MCGEOCH, D. J. (1985). DNA sequence of the region in the genome of herpes simplex virus type 1 containing the gene for the DNA polymerase and the major DNA binding protein. Nucleic Acid Res. **13,** 8143-8163.

RADY, P. L., YEN, A., ROLLEFSON, J. L., ORENGO, I., BRUCE, S., HUGHES, T. K., AND TYRING, S. K. (1995). Herpesvirus-like DNA sequences in non-Kaposi's sarcoma skin lesions of transplant patients. Lancet **345**,1339.

RAKUSANOVA, T., BEN-PORAT, T., HIMENO, M., AND KAPLAN, A. S. (1971). Early functions of the genome of herpesvirus 1, characterisation of the RNA synthesized in cycloheximide-treated infected cells. Virol. **46**, 877-889.

RAMASWAMY, R., AND HOLLAND, T. C. (1992). *In vitro* characterisation of the HSV-1 UL53 gene product. Virology **186,** 579-587.

RANDALL, C. C., ROGERS, H. W., DOWNER, D. N., AND GENTRY, G. A.. (1972). Protein kinase activity in equine herpesvirus. J. Virol. **9**, 216-222.

RANDALL, C. C., AND WALKER, B. M. (1963). Degradation of deoxyribonucleic acid and alteration of nucleic acid metabolism in suspension cultures of L-M cells infected with equine abortion virus. J. Bacteriol. **86,**138-146.

RAUH, I., AND METTENLEITER, T. C. (1991a). Pseudorabies virus glycoprotein gII and gp50 are essential for virus penetration. J. Virol **65,** 5348-5356.

RAUH, I., WEILAND, F., FEHLER, F., KEIL, G. M., AND METTENLEITER, T. C. (1991b). Pseudorabies virus mutants lacking the essential glycoprotein gII can be complemented by glycoprotein gI of bovine herpesvirus 1. J. Virol **65,** 621-631.

REA, T. J., TIMMINS, J. G., LONG, G. W., AND POST, L. E. (1985). Mapping and sequence of the gene for pseudorabies virus glycoprotein which accumulates in the medium of infected cells. J. Virol. **54,** 21-29.

REISSIG, M., AND MELNICK, J. L. (1955). The cellular charges produced in tissue culture by heptitis B virus correlated with the concurrent multiplication of the virus. J. Exptl. Med. **101,** 341-315.

RIGBY, P. W. J., DIECKMANN, M., RHODES, C., AND BERG, P. (1977). Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113,** 237-251.

RIXON, F. J., AND MCLAUCHLAN, J. (1990). Insertion of DNA sequences at a unique restriction enzyme site engineered for vector purposes into the genome of herpes simplex virus type 1. J. Gen Virol. **71**, 2931-2939.

RIXON, F. J., ATKINSON, M. A., AND HAY, J. (1983). Intranuclear distribution of herpes simplex virus type 2 DNA synthesis: examination by light and electron microscopy. J. Gen. Virol. **64**, 2087-2092.

RIXON, F. J., AND CLEMENTS, J. B. (1982). Detailed structural analysis of two spliced HSV-1 immediate-early mRNAs. Nucleic Acids Res. **10,** 2241-2256.

ROBERTSON, G. R., SCOTT, N.A., MILLER, J. M., SABINE, M., ZHENG, M., BELL, C. W., AND WHALLEY, J. M. (1991). Sequence charateristics of a gene in equine herpesvirus 1 homologous to glycoprotein H of herps simplex virus. DNA sequence **1,** 241-249.

ROBERTS, M. S., BOUNDY, A., O'HARE, P., PIZZORNO, M. C., CIUFO, D. M., AND HAYWARD, G. S. (1988). Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus 1 IE175(∂4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. **62,** 4307-4320.

ROCK, D. L., HAGEMOSER, W. A., OSORIO, F. A., AND MCALLISTER, H. A. (1988). Transcription from the pseudorabies virus genome during latent infection. Arch. Virol. **98,** 99-106.

ROCK, D. L., NESBURN, A. B., GHIASI, H., ONG, J., LEWIS, T. L., LOKENSGARD, J. R., AND WECHSLER, S. L. (1987). Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J. Virol. **61,** 3820-3826.

ROCK, D. L., AND FRASER, N. W. (1983). Detection of HSV-1 genome in central nervous system of latently infected mice. Nature **302**, 523-525.

ROIZMAN, B., AND SEARS, A. E. (1990). Herpes simplex virus and their replication, p. 1795-1894. In: B. N. fields D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Fields 'Virology", 2nd ed. Raven Press, New York.

ROIZMAN, B., CARMICHAEL, L. E., DEINHARDT, F., DE-THE, G., NAHMIAS, A. J., PLOWRIGHT, W., RAPP, F., SHELDRICK, P., TAKAHASHI, M., AND WOLF, K. (1981). Herpesviridae. definition, provisional nomenclature, and taxonomy. Intervirology **16,** 201-217.

ROIZMAN, B. (1982). The family herpesviridae: General description, taxonomy, and classification. In" The Herpesviruses", Vol 1, pp.1-23. Edited by B. Roizman, Plenun Press, New York and London.

ROIZMAN, B., AND FURLONG, D. (1974). The replication of herpesviruses. In: comprehensive Virology" Vol. 13. H. Fraenkel-Conrat and R. R. Wagner (eds). pp. 229-403 Plenum Press, N. Y. and Lond.

ROIZMAN, B., AND ROANE, P. R. (1964). The multiplication of herpes simplex virus. II. the relation between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virol. **22**, 262-269.

ROOP, C., HUTCHINSON, L., AND JOHNSON, D. C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. J. Virol. **67**: 2285-2297.

ROSALES, R., VIGNERON, M., MACCHI, M., DAVIDSON, I., XIAO, J. H., AND CHAMBON, P. (1987). *In vitro* binding of the cell-specific and ubiquitous nuclear proteins to the octamer motif of the SV40 enhancer and related motifs presented in other promoters and enhancers. EMBO J. **6**, 3015-3225.

ROSENTHAL, K., KILLIUS, J., HODNICHAK, C. M., VENETTA, T. M., GYURGYIK., L., AND JANIGA, K. (1989). Mild acidic pH inhibition of the major pathway of herpes simplex virus entry into HEp-2 cells. J. Gen. Virol. **70**, 857-867.

RüTHER, U., AND MüLLER-HILL, B. (1983). Easy identification of cDNA clones. EMBO J. **2**, 1791-1794..

RUYECHAN, W. T., AND WEIR, A. C. (1984). Interaction with nuclei acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA binding protein. J. Virol. **52,** 727-733.

RUYECHAN, W. T. (1983). The major herpes simplex virus DNA-binding protein holds single-stranded DNA in an extended configuration. J. Virol. **46,** 661-666.

RUYECHAN, W. T., DAUENHAUER, S. A., AND O'CALLAGHAN, D. J. (1982). Electron microscopy study of equine herpesvirus type1 DNA. J. Virol. **42,** 297-300.

SABINE, M., ROBERTSON, G. R., AND WHALLEY, J. M. (1981). Differentiation of subtypes of equine herpesvirus 1 by restriction endonuclease analysis. Equine Vet. J. **57**, 148-149.

SACKS, W. R., AND SCHAFFER, P. A. (1987). Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J. Virol. **61,** 829-839.

SAMBROOK, J., FRITSCH, E. F., AND MANIATIS, T. (1989). Molecular Cloning: A Laboratory Manual. 2nd ED. Cold Spring Harbour Laboratory Press. USA

SARMIENTO, M., HAFFEY, M., AND SPEAR, P. G. (1979). Membrane proteins specified by herpes simplex viruses III. Role of glycoprotein VP7 (B2) in virion infectivity. J. Virol. **29**, 1149-1158.

SAWITZKY, D., HAMPL, H., AND HABERMEHL, K. O. (1990). Comparison of heparin-sensitive attachment of pseudorabies virus (PRV) and herpes simplex virus type 1 and identification of heparin-binding PRV glycoproteins. J. Gen. Virol. **71:** 1221-1225.

SAXEGAARD, F. (1966). Isolation and identification of equine rhinopneumonitis virus (equine abortion virus) from cases of abortion and paralysis. Nord. Vet. Med. 18, 504-516.

SCHEK, N., AND BACHENHEIMER, S. L. (1985). Degradation of cellular mRNA induced by a virion associated factor during herpes simplex virus infection of vero cells. J. Virol. **55,** 601-610.

SCHERMAN, J., THOREEN, J., BARRNUM, D. A., MITCHELL, W. R., AND INGRAM, D. G. (1977). Infectious causes of equine respiratory disease on Ontario standardbred racetracks. J. Clin. Microbiol. **5,** 285-289.

SCHIMIZU, T., ISHZAKI, R., ISHII, S., KAWAKAMI, Y., KAJI, R., SUGIMURA, K., AND MATUMOTO, M. (1959). Isolation of equine abortion virus from natural cases of equine abortion in horse kidney cell culture. Japannses J. Exp. Med. **29**, 643-649.

SCHRAG, J. D., VEN KATARAM-PRASAD, B. V., RIXON, F. J., AND CHIU, W. (1989). Three dimensional structure of the herpes simplex virus 1 nucleocapsid. Cell **56,** 651-660.

SCHRODER, C. H., KAERNER, H. C., MUNK, K., AND DARAI, G. (1977). Morphological transformation of rat embryonic fibrolastis by abortive herpes simplex vrius infection : Increased transformation rate correlated to a defective viral genotype. Intervirology **8** 164-171.

SCHWARTZ, J., AND ROIZMAN, B. (1969). Concerning the egress of herpes simplex virus infected cells. Electron microscope observations. Virol. **38**, 42-49.

SEARS, A. E., HALLIBURTON, I. W., MEIGNIER, B., SILVER, S., AND ROIZMAN, B. (1985). Herpes simplex virus 1 mutant deleted in the alpha22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J. Virol. **55,** 338-346.

SEDARATI, F., IZUMI, K. M., WAGNER, E. K., AND STEVENS, J. G. (1989) Herpes simplex virus type 1 latency-associated transcription plays no role in establishment or maintenance of a latent infection in murine sensory neurones. J. Virol. **63,** 4455-4458.

SERAFINI-CESSI, F., MALAGOLLINI, N., DALL'OLIO, F., PEREIRA, L., AND CAMPADELLI-FIUME, G. (1985). Oligosaccharide chains of herpes simplex virus type 2 glycoprotein gG2. Arch. Biolchem. Biolph. **240,** 866-876.

SERAFINI-CESSI, F., DALL'OLIO, F., SCANNAVINI, M., AND CAMPADELLI-FIUME, G. (1983). Porcessing of herpes simplex virus 1 glycans in cells defective in glycosyl transferases of the Golgi system relationship to cell fusion and virion egress. Virol. **131,**59-70

SHERMAN, J., THORSEN, J., BARNUM, D. A., MITCHELL, W. R., AND INGRAM, D. G. (1977). Infectious causes of equine respiratory disease on Ontario standardbred racetracks. J. Clin. Microbiol. **5,** 285-289.

SHIDA, H., AND DALES, S. (1981). Biogenesis of vaccinia: Carbohydrate of the hemagglutinin molecule. Virol. **111,** 56-72.

SHIMIZU, T., ISHIZAKI, R., ISHII, S., KAWAKAMI, Y., KAJI, R., SUGIMURA, K., AND MATUMOTO, M. (1959). Isolation of equine abortion virus from natrual cases of equine abortion in horse kidney cell culture. Jananese J. Exp. Med. **29,** 643-649.

SLATER, J. D., BORCHERS, K., THACKRAY, A. M., AND FIELD, H. J. (1994). The trigeminal ganglion is a location for equine herpes virus 1 latency and reactivition in the horse. J. Gen. Virol. **75**, 2007-2016.

SLATER, J. D., GIBSON, J. S., AND FIELD, H. J. (1993). Pathogenicity of a thymidine kinase-deficient mutant of equine herpesvirus 1 in mice and specific pathogen-free foals. J. Gen. Virol. **74**, 819-828.

SMITH, R. H., ZHAO, Y., AND O'CALLAGHAN, D. J. (1993). The equine herpesvirus 1 (EHV1) UL3 gene, an ICP27 homologue, is necessary for full activation of gene expression directed by an EHV1 late promoter. J. Virol. **67,** 1105-1109.

SMITH, R. H., CAUGHMAN, G. B., AND O'CALLAGHAN, D. J. (1992). Characteriaztion of the regulation function of the equine herpesvirus 1 immediate-early gene product. J. Virol. **66**, 936-945.

SMITH, R. F., AND SMITH, T. F. (1989). Identification of new protein kinase-related genes in three herpesvirus, herpes simplex virus, varicella zoster virus, and Epstein-Barr virus. J. Virol. **63**, 450-455.

SMITH, J. D., AND DE HARVEN, E. (1974). Herpes simplex virus and human cytomegalovirus replication in W1-38 cells. II. An ultrastructural study of viral penetration. J. Virol. **14**, 945-956.

SPEAR, P. G. (1993 a). Entry of alphaherpesviruses into cells. Seminars in Virology, **4,** 167-180.

SPEAR, P. G. (1993 b). Membrane fusion induced by herpes simplex virus fusion mechanisms (eds by Bentz J.), pp. 201-232. CRC Press. Boca. Raton FL.

SPEAR, P. G., SHIEH, M. T., HEROLD, B. C., WUDUNN, D., AND KOSHY, T. (1992). Heparan sulfate glycosaminoglycans as primary cell surface receptors for herpes simplex virus. Adv. Exp. Med. Biol. **33**:341-353.

SPEAR, P. G. (1984). Glycoprotein specified by herpes simplex viruses. p315-356. In: B. Roizman (ed.) The herpesviruses. Vol.3 Plenum Publishing Corp., New York.

HEINE, J.W., SPEAR, G. P., AND ROIZMAN, B. (1972). Proteins specified by herpes simplex virus. VI. Viral proteins in plasm membrane. J. Virol. **9**, 431-439.

SPIVACK, J. G., AND FRASER, N. W. (1987). Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J. Virol. **61,** 3841-3847.

STACKPOLE, C. W. (1969). Herpes-type virus of the frog renal adenocarcinoma. I. Virus development in tumour transplants maintained at low temperature. J. Virol. **4,** 75-93.

STEINER, I., SPIVACK, J. G., DESHMANE, S. L., ACE, C. I., PRESTON, C. M., AND FRASER, N. W. (1990) A herpes simplex virus type 1 mutant containing a non-transinducing Vmw65 protein establishes latent infection *in vivo* in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. J. Vriol. **64,** 1630-1638.

STEINER, I., SPIVACK, J. G., LIRETTE, R. P., BROWN, S. M., MACLEAN, A. R., SUBAK-SHARPE, J. H., AND FRASER, N. W. (1989). Herpes implex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. **8,** 505-511

STEVENS, A. C., ROBERTS, C. R., HAY. J., BISHER, M. E., PUN, T., AND TRUS, B. L. (1986). Hexavalent capsomers of herpes simplex virus type 2: symmetry, shape, dimensions, and oligomeric status. J. Virol. **57**, 578-384.

STEVENS, J. G., HAARR, L., PORTER, D. D., COOK, M. L., WAGNER, E. K. (1988). Prominence of the herpes simplex virus latency-associated transcript in trigeminal ganglia from seropositive humans. J. Infect. Dis. **158,** 117-123.

STEVENS, J. G., WAGNER, E. K., DEVI-RAO, G. B., COOK, M. L., AND FELDMAN, L.T. (1987). RNA complementary to a herpesvirus α mRNA is predominent in latantly infected neurons. Science **235,** 1056-1059.

STEVENS, J. G., COOK, M. L., AND JORDAN, M. C. (1975). Reactivation of latent herpes simplex virus after pneumonococcal pneumonia in mice. Infect. Immun. **11,** 635-638.

STOLLAR, V. (1979). Defective interfering particles of togaviruses. In current topics in Microbiology and Immunology, Vol. 86, pp. 35-66. Edited by. Arber, et al. Borlin and Heidelberg: Springer Verlag.

move after STOKES, A., ...

STOKES, A., ALBER, D. G., GREENSILL, J., AMELLAL, B., CARVALHO, R., TAYLOR, L. A., DOEL, T. R., KILLINGTON, R. A., HALLIBURTON, I. W., AND

MEREDITH, D. M. (1996). The expression of the proteins of equine herpesvirus 1 which share homology with herpe simplex virus 1 glycoproteins H and L. Virus Research **40** 91-107.

STOKES, A., ALLEN, G. P., PULLEN, L. A., AND MURRAY, P. K. (1989). A hamster model of equine herpesvirus type 1 (EHV1) infection; Passive protection by monoclonal antibodies to EHV1 glycoproteins 13, 14 and 17/18. J. Gen. Virol. **70,** 1173-1183.

STOKER, M. G. P., SMITH, K. M., AND ROSS, R. W. (1958). Electron microscopic studies of Hela cells infected with herpes virus. J. Gen. Microbiol. **19,** 244-249
protein. Vriol. **196,** 413-418.

STORZ, J., EHLERS, B., TODD, W. J., AND LUDWIG, H. (1984). Bovine cytomegaloviruses: Identification and differential properties. J. Gen. Virol. **65,** 697-706

STOW, N. D., HUMMARSTEN, O., ARBUCKLE, M. I., AND ELIAS, P. (1993). Inhibition of herpes simplex virus type 1 DNA replication by mutant forms of the origin-binding protein. Virol. **196**, 413-418.

STOW, N. D. (1992). Herpes simplex virus type 1 origin-dependent DNA replication in insect cells using recombinant baculoviruses. J. Gen. Vriol. **73**, 313-321.

STOW, N. D., AND DAVISON, A. J. (1986). Identification of a varicella-zoster virus origin of DNA replication and its activation by herpes simplex virus type 1 gene products. J. Gen. Virol. **67**, 1613-1623.

STOW, N. D., MURRAY, M. P., AND STOW, E. C. (1986). Cis-acting signals involved in the replication and packaging of herpes simplex virus type 1 DNA. In: cancer cells/4: DNA Tumour viruses pp. 497-567. Edited by M. Botchan, T. Grodzicker and P. A. Sharp. New. York: Cold Spring Harbor Laboratory USA.

STOW, N. D. (1982). Localization of an origin of DNA replication within the TRs/IRs repeated region of the herpes simplex virus type 1 genome. EMBO J. **1**, 863-867.

STOW, N. D., AND WILKIE, N. M. (1976). An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. J. Gen. Virol. **33**, 447-458.

STUDDERT, M. J., SIMPSON, T., AND ROIZMAN, B. (1981). Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. Science, **214,** 562-564.

STUDDERT, M. J. (1983). Restriction endonuclease DNA finger-printing of respiratory, foetal and perinatal foal isolates of equine herpesvirus type 1. Arch. Virol. **77,** 249-258.

STURM. R. A., AND HERR, W. (1988). The POU domain is a bipartite DNA-binding structure. Nature **336**, 601-604.

SULLIVAN, D., ALLEN, G. P., AND O'CALLAGHAN, J. D. (1989). Synthesis and processing of equine herpesvirus type 1 glycoprotein 14. Virology **173,** 638-646.

SUN, Y., MACLEAN, A. R., AITKEN, S. D., AND BROWN, S. M. (1996). The role of the gene 71 product in the life cycle of equine herpesvirus 1. J. Gen. Virol. **77,** 493-500.

SUN, Y., AND BROWN, S. M. (1994). The open reading frames 1, 2, 71 and 75 are non-essential for replication of equine herpesvirus type 1 *in vitro*. Virol. **199,** 448-452.

SUN, Y., MACLEAN, R. A., DARGEN, D., AND BROWN, S. M. (1994). Identification and characterisation of the protein product of gene 71 in equine herpesvirus 1. J. Gen. Virol. **75,** 3117-3126.

SYDISKIS, R. J., AND ROIZMAN, B. (1967). The disaggregation of host polyribosomes in productive and abortive infection with herpes simplex virus. Virol. **32**,678-686.

SZILáGYI, J. F., AND CUNNINGHAM, C. (1991) Identification and characterization of a novel noninfectious herpes simplex virus-related particle. J. Gen. Virol. **72,** 661-668.

TAHA, M. Y. (1991). Analysis of neurovirulence in the mouse model system using deletion variants of herpes simplex virus type 2 (HSV-2). Ph.D. thesis, University of Glasgow.

TELFORD, E. A. R., STUDDERT, M. J., AGIUS, C. T., WATSON, M. S., AIRD, H. C., AND DAVISON, A. J. (1993). Equine herpesvirus 2 and 5 are gamma-herpesviruses, Virol. **195**, 492-499.

TELFORD, E. A. R., WATSON, M. S., MCBRIDE, K., AND DAVISON, A. J. (1992). The DNA sequence of equine herpesvirus 1. Virol. **198,** 304-316.

TEWARI, D., WHALLEY, J. M., LOVE, D. N., AND FIELD, H. J. (1994). Characterization of immune responses to baculovirus-expressed equine herpesvirus type 1 glycoproteins D and H in a murine model. J. Gen, Virol. **75,** 1735-1741.

TEWARI, D., GIBSON, J. S., SLATER, J. D., O'NEILL, T., HANNANT, D., ALLEN., G. P., AND FIELD, H. J. (1993). Modulation of the serological response of specific pathogen-free (EHV-free) foals to EHV1 by previous infection with EHV-4 or a TK-deletion mutant of EHV1. Arch. Virol. **132,** 101-120.

THOMPSON, G. R. (1978). The role equine herpesvirus 1 in acute respiratory disease of horses and the use of inactivated antigens to immunise against the infection. Ph.D. Thesis, University of London.

TIKOO, S. K., FITZPATRICK, D. R., BABIUK, L. A., AND ZAMB, T. J. (1990). Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. J. Virol **64,** 5132-5142.

TURTINEN, L. W. (1983). Studies on the antigenic and genetic herpesvirus 1 Ph. D. Dissertation, University of Kentucky.

TURTINEN, L. W., AND ALLEN, G. P. (1982). Identification of envelope surface glycoproteins of equine herpesvirus type 1. J. Gen. Virol. **63**, 481-485.

VERRIJZER, C. P., KAL, A. J., AND VAN DER VLIET, P. C. (1990). The DNA binding domain (POU domain) of transcription factor Otc-1 suffices for stimulation of DNA replication. EMBO J. **9** 1883-1888.

VON HEIJNE, G. (1986). A new method for predicting signal-sequence cleavage sites. Nucleic Acids Res. **14**, 4683-4690.

VON HEIJNE, G. (1983). Patterns of aminao acids near signal-sequence cleavage sites. Eur. J. Biochem. **133,** 17-21.

VON MAGNUS, P. (1951). Propagation of the pr-8 strain of influenza A virus in chick embryos. III. Properties of the incomplete virus produced in serial passages of undiluted virus. Acta Pathol. Microbiol. Scand. **29**, 157-188.

VON STEINHAGEN, P. (1988). Zur situation der equinen herpesvirus type 1 (EHV1)-infecktion in der warmblutzuchi schleswig-holsteins. Tierärztliche Umschau **43,** 348-349.

WAGENAAR, F., POL, J. M. A., PEETERS, B., GIELKENS, A. L. J., de WIND, N., AND KIMMAN, T. G. (1995). The Us3-encoded protein kinase from pserdorabies virus affects egress of virions from the nucleus. J. Gen. Virol. **76,** 1851-1859.

WAGNER, E. K., FLANAGAN, W. M., DEVI-RAO, G., ZHANG, Y.-F., HILL, J. M., ANDERSON, K. P., AND STEVENS, J. G. (1988). The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. J. Virol. **62,** 4577-4585.

WAGNER, E. R. (1985). Individual HSV transcripts. In: "The Herpesviruses", Vol. 3, p. 45-104.. B. Roizman, (ed), Plenum. Publishing Corp. New York.

WAGNER, E. K., SHARP, J. A., AND SUMMERS, W. C. (1981). Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type 1. Pro. Natl. Acad, Sci. USA **78,** 1441-1445.

WALLENFELS, B. (1979). Developmental and mutational changes of glycoproteins in the mouse neuronal retina: studies with bovine galactosyl transferase. Proc. Natl. Acad. Sci USA, **76,** 3223-3227.

WALZ, M. A., PRICE, R. W., AND NOTKINS, A. L. (1974). Latent ganglionic infection with herpes simplex virus type 1 and 2 : viral reactivation *in vivo* after neurectomy. Science **184,** 1185-1187.

WARREN, K. G., BROWN, S. M., WROBLEWSKA, Z., AND SUBAK-SHARPE, J. H. (1978). Isolation of latent herpes simplex virus from the superior cervical and vagus ganglions of human beings. N. Engl. J. Med. **298,** 1068-1069.

WATHEN, M. W., AND STINSKI, M. F. (1982). Temperal patterns of human Cytomegalovirus transcription: mapping the viral RNAs synthesised at immediate early, early and late times after infection. J. Virol. **41,** 462-477.

WEBER, P. C., LEVINE, M., AND GLORIOSO, J. C. (1987). Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science **236,** 576-579.

WEINHEIMER, S. P., AND MCKNIGHT, S. L. (1987). Transcriptional and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. J. Mol. Biol. **195**, 819-833.

WEIR, H. M. , AND STOW, N. D. (1990). Two binding sites for the herpes simplex virus type 1 UL9 protein are required for efficient activity of the replication origin. J. Gen. Virol. **71**, 1379-1385.

WELCH, H. M., BRIDGES, C. G., LYON, A. M., GRIFFTTHS, L., AND EDINGTON, N. (1992). Latent equid herpesviruses 1 and 4: detection and distinction using the polymease chain reaction and co-cultivation from lymphoid tissues. J. Gen. Virol. **73,** 261-268.

WECHSLER, S. L., NESBURN, A. B., ZWAAGSTRA, J., AND GHIASI, H. (1989). Sequence of the latency-related gene of herpes simplex virus type 1. Virology **168,** 168-172.

WHALLEY, J. M., ROBERTSON, G., BELL, C., LOVE, D., ELPHINSTONE, M., WILEY, L., AND CRAVEN, D. (1991). Identification and comparative sequence analysis of a gene in equine herpesvirus 1 with homology to the herpes simplex virus glycoprotein D gene. Virus Genes, **5,** 313-325.

WHALLEY, J. M., ROBERTSON, G. R., SCOTT, N. A., HUDSON, G. C., BELL, C. W., AND WOODWORTH, L. M. (1989). Identification and nucleotide sequence of a gene in equine herpesvirus 1 analogous to the herpes simplex virus gene encoding the major envelope glycoprotein gB. J. Gen. Virol. **70,** 383-394.

WHALLEY, J. M., ROBERTSON, G. R., AND DAVISON, A. J. (1981). Analysis of the genome of equine herpesvirus type 1: arrangement of cleavage sites for restriction endonucleases EcoRI, BglII and BamHI. J. Gen. Virol. **57,** 307-323.

WHEALY, M. E., CARD, J. P., MEADE, R. P., ROBBINS, A. K., AND ENQUIST, L. W. (1991). Effect of Brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J. Virol. **65** 1066-1081.

WHITLEY, R. J. (1985). Epidemiology of herpes simplex viruses. In: "The herpesviruses", Vol, 3, pp 1-44. Roizman, b., (Ed.) Plenum Press. New York and London.

WHITTAKER, G. R., BONASS, W. A., ELTON, D. M., HALLIBURTON, I. W., KILLINGTON, R. A., AND MEREDITH, D. M. (1992a). Glycoprotein 300 is encoded by gene 28 of equine herpesvirus type 1: a new family of herpesvirus membrane proteins? J. Gen. Virol. **73,** 2933-2940.

WHITTAKER, G. R., TAYLOR, L. A., ELTON, D. M., AND MEREDITH, D. M. (1992b). Glycoprotein 60 of equine herpes virus type 1 is a homologue of herpes simplex

virus type 1 glycoprotein D and plays a major role in penetration of cells. J. Gen. Vriol. **73**, 801-809.

WHITTAKER, G. R., WHELDON, L. A., GILES. L. E., STOKES, J. M., HALLIBURTON, I. W., KILLINGTON, R. A., AND MEREDITH, D. M. (1990). Characterization of the high Mr glycoprotein (gp300) of equine herpesvirus type 1 as a novel glycoprotein with extensive O-linked carbohydrate. J. Gen. Virol. **71**, 2407-2416.

WHITTON, J. L., AND CLEMENTS, J. B. (1984). Replication origins and a sequence involved in coordinate induction of the immediate-early gene family are conserved in an intergenic region of herpes simplex virus type 1. Nucl. Acids Res. **11,** 6271-6278.

WILLIAMS, M. V. (1988). Herpes simplex virus-induced dUTPase: Target site for antiviral chemotherapy. Virol. **166**, 262-264.

WILCOX, C. L., SMITH, R. L., FREED, C. R., AND JOHNSON, E. M. (1990). Nerve growth factor dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons *in vitro.* J. Neuro. Sci. **104,** 1268-1275.

WILCOX, C. L., AND JOHNSON, E. M. (1988). Characterization of nerve growth factor-dependent herpes simplex virus latency in neurons *in vtro.* J. Virol. **62,** 393-399.

WILDY, P., FIELD, H. J., AND NASH, A. A. (1982). Classical herpes latency revisited. In "Virus Persistence Symposium 33, society of General Microbiology", pp. 133-168. Edited by W. J. MAHY, A. C. MINSON AND G. K. daMay. Cambridge University Press.

WILDY, P., RUSSELL, W. C., AND HORNE, R. W. (1960). The morphology of herpes virus. Virol. **12**. 204-222.

WILKIE, N. M. (1973). The synthesis and substructure of herpesvirus DNA: The distribution of alkali-labile single strand interruptions in HSV1-DNA. J. Gen. Virol. **21,** 453-467.

WILKS, C. R., AND COGGINS, L. (1977). Protective immunity in equine herpesvirus type 1 infection of hamsters. Cornell veterinarian **67,** 385-403.

WILSON, D. W., DAVIS-POYWTER, N., AND MINSON, A. C., (1994). Mutation in the cytoplamic tail of herpes simplex virus glycoprotein H suppress cell fusion by a syncytial strain. J. Virol. **68**, 6985-6993.

WU, C. A., NELSON, N. J., MCGEOCH, D J., AND CHALLBERG, M. D. (1988). Identification of herpes simplex virus type 1 gene required for origin-dependent DNA synthesis. J. Virol. **62,** 435-443.

WUDUNN, D., AND SPEAR, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. **63,** 52-58.

YALAMANCHILI, R., AND O'CALLAGHAN, D. J. (1990). Sequence and organization of the genomic termini of equine herpesvirus tpe 1 . Viurs Res. **15**, 149-162.

YAMANISHI, K., OKUNO, T., SHIRAKI, K., TAKAHASHI, M., KONDO, T. , ASANO, Y., AND KURATA, T. (1988). Identification of human herpes virus-6 as a causal agent for exanthem subitum. Lancet **i**,1056-1067.

YANISCH-PERRON, C., VIEIVA J., AND MESSING, J. (1985). Improved MB phage cloning vectors nucleotide sequences of the M13 18 and pUC19 vector. Gene **103**, 1985.

YASWEN, L. R., STEPHENS, E. B., DAVENPORT, L. C., AND HUTT-FLETCHER, L. M. (1993). Epstein-Barr virus glycoprotein gp85 associates with the BHRF2 gene product and is incompletely processed as a recombinant protein. Virology **195,**387-396.

ZHAO, Y., HOLDEN, V. R., SMITH, R. H., AND O'CALLAGHAN, D. J. (1995). Regulatory function of the equine herpesvirus 1 ICP27 gene protein. J. Virol. **69,**2786-2793.

ZHAO, Y., HOLDEN, V. R., HARTY, R. N., AND O'CALLAGHAN, D. J. (1992). Identification and transcriptional analysis of the UL3 and UL4 genes of equine herpes virus 1, homologies of the ICP27 and glycoprotein K genes of herpes simplex virus. J. Virol. **66**, 5363-5377.

ZWAAGSTRA, J. C., GHIASI, H., NESBURN, A. B., AND WECHSLER, S. L. (1991). Identidication of a major regulatory sequence in the latency associated transcript (LAT) promoter of herpes simlex virus type 1 (HSV-1). Virology **182**, 287-297.

GLASGOW UNIVERSITY LIBRARY