Studies on some determinants of virulence in *Alcelaphine herpesvirus*-1

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DECLARATION

The work described in this thesis was carried out at the Moredun Research Institute, Edinburgh and the National veterinary Research Institute, Muguga , Kenya.

The initiation, design and execution of the experimental work and the interpretation of the results presented in this thesis was carried out by the author. Contributions to the work in this thesis by colleagues is fully acknowledged in the text.

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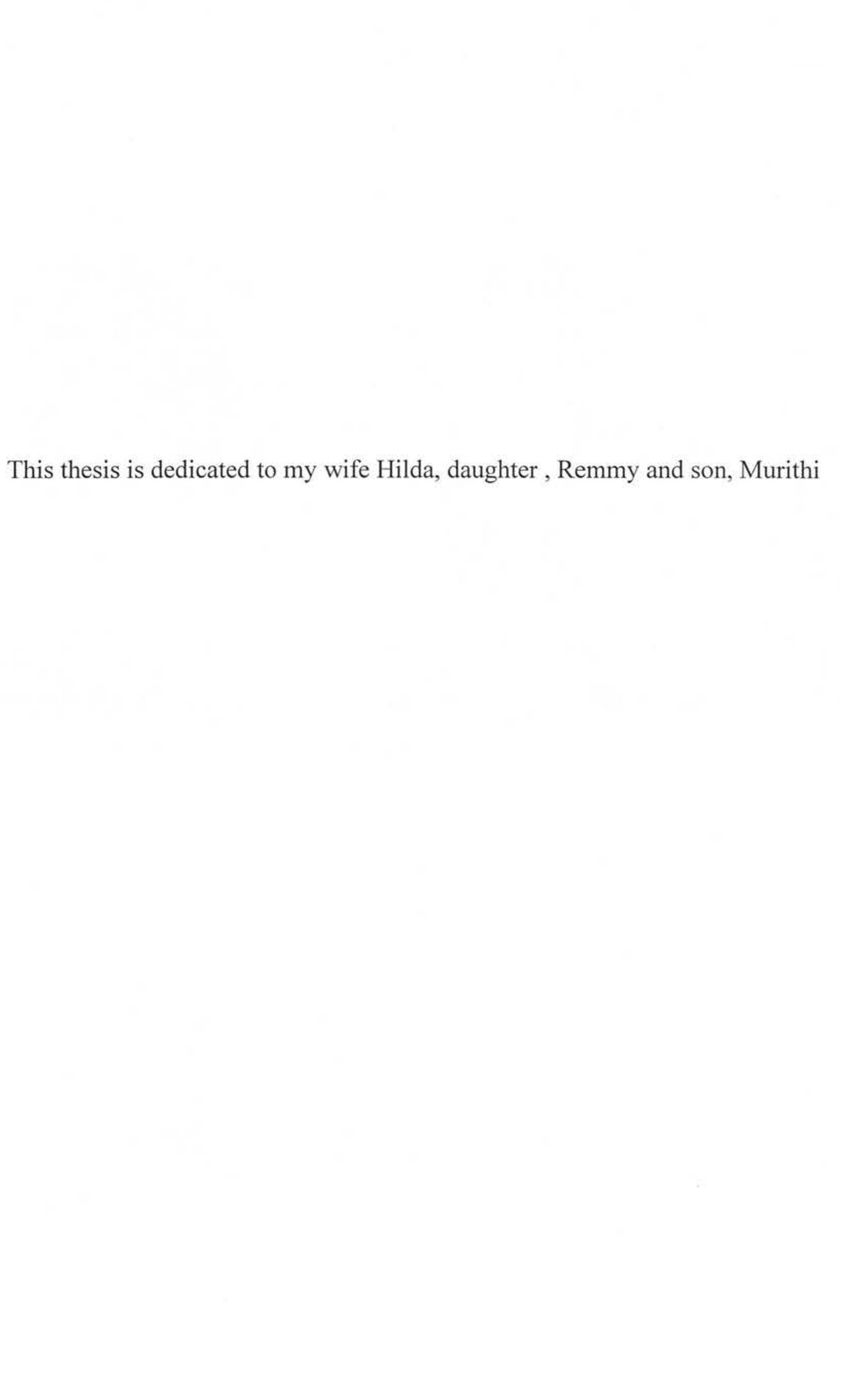

This thesis is dedicated to my wife Hilda, daughter , Remmy and son, Murithi

ABSTRACT

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative disease of Artiodactyla. The disease is caused by infection of susceptible hosts with one of two gammaherpesviruses, Alcelaphine herpesvirus-1 (AHV-1) or Ovine herpesvirus-2 (OHV-2).

On primary isolation, AHV-1 infectivity is cell-associated and the virus can induce MCF following inoculation into susceptible hosts. Cell free virus which is pathogenic for cattle is observed following low serial passage of the virus in cell cultures. After further serial passage cell free virus is observed, but this virus cannot produce disease experimentally. AHV-1 genomic rearrangements occur during the transition from virulence to attenuation. Two genes, encoding putative protein 5 (P-5) and protein 1 (P-1), are truncated during this rearrangement. The aim of this study was determine the importance of these putative proteins in the pathogenesis of AHV-1.

Sequence encoding each of these proteins was cloned and the proteins expressed *in vitro*. Rabbits, a laboratory model for AHV-1, were successfully immunised with these proteins. Neither of these proteins however induced a protective immune response. Although both proteins are expressed *in vitro*, transcripts for these proteins could not be detected *in vivo* in animals reacting with MCF.

During the course of this study, the complete sequence of the AHV-1 genome was published. P-1 and P-5 were shown to form part of open reading frames (ORF) 50 and A7 respectively. These ORFs were therefore re-assessed to determine their positions in the attenuated and virulent virus. New isolates were obtained from cattle showing clinical MCF and also from wildebeest. Analysis of viral DNA derived from the new

isolates has shown that ORF 50 and ORF A7 are conserved between isolates and the virulent laboratory adapted isolate. The results presented here show that a block of sequence in the attenuated virus, which includes sequence for ORF 50 and its promoter, are translocated from a position in the middle of the unique DNA to a terminal position where it is inverted with respect to its transcriptional orientation in the virulent virus. The ORF is transcriptionally silent in the attenuated virus and the results presented here show that this is due to the inactivity of the truncated ORF 50 promoter in the attenuated virus.

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Abbreviations

APMSF	Aminophenylmethylsulphonylfluoride
BCIP	5-bromo-4-chloro-3-indolylphosphate
BPB	Bromophenol Blue
BSA	Bovine serum albumin
cpe	Cytopathic effect
DIG	Digoxygenin
DMEM	Dulbecco's modified Eagle's medium
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GST	Glutathione-S-transferase
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
IPA	Isopropanol
IPTG	Isopropyl-b-D-thiogalactoside
kbp	kilobase pairs
kDa	kilodaltons
LB	L-broth.
LCL	Large cytotoxic lymphocyte
MOPS	(3-[N-Morpholino]propanesulphonic acid)
MRI	Moredun Research Institute
NBT	Nitroblue tetrazolium
NVRC	National Veterinary Research Laboratory, Muguga
OD	Optical density
OPD	Orthophenyl Diamine
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride/sodium citrate
ssDNA	Single stranded DNA
TAE	Tris/Acetate/EDTA
TBS	Tris-buffered saline
TE	10mM Tris/0.1mM EDTA
TEMED	N,N,N,N-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
TRX	Thioredoxin
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

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.CHAPTER 1

INTRODUCTION

1.1 General introduction

Malignant catarrhal fever (MCF) is a clinico-pathologic syndrome of Artiodactyla characterised by fever and high mortality but generally low morbidity, worldwide distribution and defined by characteristic histopathologic changes (Plowright 1986, Locken et al, 1998). MCF has been recognised as a sporadic disease of cattle since the early part of this century (Goetze, 1930) but outbreaks have also been reported in cattle and deer (Daubney and Hudson, 1936, Plowright, 1968, Reid et al, 1979). Cases of MCF have been reported in at least 39 species of Bovidae and Cervidae (Reid and Buxton, 1984, Heuschele et al, 1988 a,b, Metzler 1991) and recently in farmed and exotic pigs in Norway (Locken et al, 1998).

In regions of Africa the disease is economically important as it affects cattle. In Asia, the water buffalo (*Bubalus bubalis*) and banteng cattle (*Bos javanicus*) are particularly susceptible and the disease has become more important in the UK, New Zealand and Australia following the development of deer farming (Denholm and Westbury, 1982, Reid et al, 1984). In Africa, the disease is caused by infection with a gammaherpesvirus, Alcelaphine herpesvirus 1 (AHV-1) which is readily isolated from affected cattle and inapparently affected wildebeest (*Connochaetes taurinus*), (Plowright et al, 1960). In other parts of the world the disease is associated with Ovine herpesvirus 2 (OHV-2) which has not been isolated but sheep are the natural hosts of the virus (Rossiter 1980b, Baxter et al, 1993). The disease can be caused by either of the two viruses. These viruses are antigenically and genetically related and the disease picture following infection of susceptible hosts with either aetiological agent is generally similar.

1. 2. The disease

1. 2. 1. Clinical disease

The clinical features of MCF are variable and have been categorised into; peracute, acute head and eye form, intestinal, mild and chronic forms (Plowright 1968). The majority of cases in cattle are acute while in deer the peracute form is the most common (Plowright 1986). Following an incubation period which may vary from a few weeks to several months, the animal is usually noticed as being dull and inappetant while the milk yield in lactating animals rapidly drops. This is generally followed by lacrimation, salivation and secretion of watery nasal discharge which progresses to become profuse and mucopurulent (Plowright 1968, Selman et al, 1974). This is followed a few days later by severe congestion, necrosis and erosion of the nasal and ocular mucosae (Reid and Buxton 1984, reviewed by Plowright, 1986, and see figure 1.1). Lacrimation sometimes becomes mucopurulent and is accompanied by centripetal corneal opacity and blindness. There is generalised enlargement of superficial lymph nodes and this may occur several days before onset of fever. Less frequent signs include laminitis and dermatitis and these have only been seen in disease associated with OHV-2 (Plowright, 1986). An unusual case of MCF in a yearling bull where the only symptoms seen were lameness and exudative dermatitis which resulted in multiple skin fissures has also been reported (Holiman et al, 1994). Neurological symptoms are not uncommon and are characterised by muscular tremors, incoordination, hyperaethesia and head pressing (Daubney and Hudson, 1936, Selman et al, 1974). The disease in deer is often hyperacute with a

short course and the clinical picture may be less dramatic than in cattle. The disease in deer may include slight lymphadenopathy, mild congestion of the buccal mucosae, slight nasal discharge and diarrhoea (Oliver et al, 1983, Reid and Buxton, 1984) or it may be acute as observed in cattle (Westbury and Denholm, 1982).

Experimental transmission is only possible with suspensions of viable cells prepared from affected tissues.

The disease is experimentally reproducible in cattle and rabbits following inoculation with AHV-1 though less readily with OHV-2 and the symptoms seen are largely similar to those seen in natural cases. The average incubation time in experimentally infected cattle is 30 days for AHV-1 and 32 days for OHV-2 (Piercy et al, 1952a, 1952b). The course of the disease lasted 12 days in cattle infected with AHV-1 and only 4 days in OHV-2 affected animals. In rabbits the disease has an incubation period of 11- 12 days (Piercy 1955, Rossiter 1981). In rabbits inoculated intracerebrally there is sudden onset of fever which subsides in 2-3 days. Death usually occurs 2 to 3 days after fever without any other symptoms. In some rabbits however symptoms appear during pyrexia and these are characterised by "paddling", "circling", muscular tremors, incoordination and paralysis. In rabbits that are inoculated by the intraperitoneal route or intravenously, the animals may become pyrexic and then die without any other signs but some may show ocular and nasal discharges which may become mucopurulent. Other signs include mucoid diarrhoea and enlarged peripheral lymph nodes (Piercy, 1955).

Figure 1.1; Typical case of a bull affected with the head and eye form of MCF. Note the oral, nasal, muzzle erosions and oral discharge and closed eyes

1. 2. 2. Epizootiology

1. 2. 2.1. Natural hosts

The close association between MCF in cattle and the wildebeest calving season has been recognised since the last century, (Cummings, 1850, Mettam, 1923). The virus was however isolated later from normal pregnant wildebeest and yearling calves (Plowright et al, 1960). Although some wildebeest calves acquire infection congenitally, transmission is thought to be mainly horizontal from contaminated pastures during the neonatal period (Plowright et al, 1965a,b , Mushi et al, 1980b, 1981a). In infected calves the virus most probably replicates in the corneal and turbinate mucosa from where it is shed in a cell free form (Mushi et al, 1981c). Virus shedding continues until the calves are about 3 months old following production of surface IgA and appearance of virus neutralising antibody in the blood (Plowright, 1965, Mushi et al, 1981c, Rurangirwa et al, 1982,).

AHV-1 has been isolated from adult wildebeest following treatment with corticosteroids or under the stress of confinement, malnutrition or pregnancy (Plowright, 1965, Rweyemamu et al, 1974). The disease in Africa is therefore observed during and immediately after the wildebeest calving season (Plowright, 1965, Mushi et al, 1981c) and the disease in zoological collections has been shown to follow the same pattern (Heuschele et al, 1988a,b).

Most cases of OHV-2 infection are associated with close contact between sheep and clinically susceptible hosts, particularly during the lambing season, indicating that

lambs play a similar role to that played by wildebeest calves (Plowright, 1986). Polymerase chain reaction test (PCR) has shown that virus is present in colostrum of suckling ewes and lambs up to 12 weeks of age (Li et al, 1995b) and that all adult sheep tested had antibody to OHV-2 (Rossiter, 1982). The PCR has now been used to detect OHV-2 DNA in nasal secretions of lambs within a period of two months following birth. Viral DNA was also detected in epithelial cells of the cornea, turbinates and pharynx which led to the proposal that the cornea and turbinates are the primary sources of OHV-2 infection in lambs (Baxter et al, 1997). Perinatal infection of lambs is low but infection rates rise rapidly after nursing indicating that the major source of OHV-2 infections is milk (Li et al, 1998).

1. 2. 2. 3. Other possible hosts

Serological studies have shown prevalence of antibody in at least 33 species within the subfamilies Alcelaphinae, Hippotraginae, Caprinae and Reduncinae (Hamblin and Hedger, 1984, Heuschele et al, 1984, Metzler, 1991). Whether the antibody is a response to AHV-1, OHV-2 or antigenically related viruses is unclear. However distinct but antigenically related viruses have been isolated from the hartebeest, (*Alcelaphus buselaphus*) (Reid and Rowe, 1973), topi (*Damaliscus korrigum)* (Mushi et al, 1981b) and the roan antelope (*Hippotragus equinus*) (Reid and Bridgen, 1991). These viruses though present in the natural host at a high prevalence do not appear to spread to other species and cause MCF.

1. 2. 2. 4. Transmission

The most probable source of AHV-1 infection for cattle is infective ocular and nasal secretions from wildebeest calves (Mushi et al, 1980a, b, 1981c). Infected cattle are dead-end hosts and this is believed to be due to cattle shedding cell-associated virus (Mushi et al, 1981c). The method of transmission for OHV-2 has not been elucidated though it is believed to be similar to that observed with AHV-1. PCR has shown that OHV-2 is present in B lymphocytes of adult sheep and that lambs may acquire virus from milk (Li et al, 1995b, Baxter et al, 1997). The virus then replicates in the upper respiratory mucosae from where the virus is shed via nasal secretions (Baxter et al, 1997). Experimental transmission can be demonstrated using blood or cell suspensions of tissues from cattle, rabbits and deer affected with either of the two viruses (Daubney and Hudson 1936, Selman et al, 1974, Buxton and Reid, 1980, Westbury and Denholm 1982). Transmission to hamsters has also been achieved with OHV-2 and the C500 isolate of AHV-1 while only AHV-1 would transmit to rats (Reid et al, 1986 Jacoby et al, 1988a)

1. 2. 3 Pathology

1. 2. 3. 1 Gross pathology

Although the general pathology of MCF was first reported by Daubney and Hudson in 1936, detailed systematic pathology was described later by Plowright (1968) and Selman et al, (1974, 1978) following natural and experimental infection of cattle with

AHV-1 and OHV-2 respectively. The pathology in deer is similar to that of cattle following infection with either virus (Huck et al, 1961, Plowright, 1968). MCF is a disseminated disease and lesions are found in all organs and tissues. In the alimentary system the lesions may vary from severe congestion to erosions and necrotic patches in the buccal and pharangeal mucosae; the abomasal and intestinal mucosae may be reddened or haemorrhagic (figure 1.2) and button ulcers have been observed in abomasal folds in some chronic cases of MCF (Daubney and Hudson, 1936, Plowright, 1968, Selman et al, 1974). The peripheral and mesenteric lymph nodes are enlarged, soft and they may be haemorrrhagic and oedematous or necrotic (Plowright 1968, Selman 1974 Westbury and Denholm, 1982). The spleen is markedly enlarged with prominent white pulp (Plowright, 1968). Lesions in the respiratory system are severe in animals affected with AHV-1 and are characterised by congestion, haemorrhage and necrosis of the upper respiratory tract but involvement of the lungs is rare. In the parenchymatous organs, the liver is usually congested and enlarged (Daubney and Hudson, 1936) and the kidney lesion is characterised by cortical infarcts which are surrounded by areas of haemorrhage.

Figure 1.2; Typical appearance of the gut in a steer with the head and eye form of MCF

Note the hyperemia and ulcers in the intestinal mucosa

1. 2. 3. 2 Histopathology

The most striking histopathological feature of MCF is infiltration of all organs with mononuclear cells and the presence of vascular lesions (Selman et al, 1974). Multifocal, degenerative and necrotic changes are observed in all respiratory and digestive system mucous membranes and squamous epithelia in AHV-1 or OHV-2 affected animals and these are associated with lymphocytic infiltration (Selman et al, 1974, Liggitt et al, 1978, Liggitt and DeMartini, 1980b, c). In the kidneys, foci of mononuclear cell infiltration correspond to the grey-foci seen macroscopically although there is development of infarcts as a result of necrotising arteritis and thrombosis. The vessels affected are mainly the small and medium arteries of most tissues, the most commonly affected being the oro-pharangeal epithelium, eye, nose, kidney, liver, lung and brain. Ultrastructural studies showed that the infiltrative cells were mainly lymphocytes, lymphoblasts and macrophages (Liggit and DeMartini, 1980b, c). lymphoid cell accumulations in lesions carry T-cell surface markers (Schock, 1996). In recovered animals or chronically affected cattle, chronic obliteropathic lesions are seen with infiltrative cells predominantly found in the tunica media and intima resulting in the occlusion of blood vessels (O'Toole et al, 1995).

1. 2. 3. 3 Pathogenesis

Following infection with AHV-1 by inoculation of cattle with infective blood,

viraemia occurs after 8-17 days (Plowright, 1968). The first overt sign of disease however is pyrexia which is observed 3-5 days after viraemia. The virus then appears in the lymphoid organs before it becomes disseminated. Pathological changes then occur in all organs and tissues and death usually occurs 5-8 days after the onset of pyrexia. Work done using rabbits inoculated with OHV-2 (Buxton and Reid 1980) showed that earliest changes are characterised by thickening of the appendix (a lymphoid organ in lagomorphs). This is followed 2 days later by enlargement of superficial lymph nodes and the mesenteric lymph nodes, spleen, and alveolar emphysema. Edington and Patel (1981) inoculated rabbits intravenously with AHV-1 infective tissue suspensions and showed that the primary target organ for the virus was the spleen. They also suggested that the secondary target sites were the lymphoid glands.

One striking feature of MCF however is the lack of direct virus cytopathology (Plowright 1986). Although virus infectivity can be readily demonstrated in tissues, no virus specific antigens have been detected (Rossiter, 1980a) and DNA hybridisation can detect only 1 out of 10^4 infected cells in affected tissues (Bridgen et al, 1992).

There are generally three major processes of cellular damage in virus infections. These processes are direct cellular damage due to virus replication, cell transformation, and pathology due to immune activation or suppression following infection of cells by virus. All these processes however involve various viral molecular determinants of virulence.

Earlier theories suggested that the pathology in MCF observed was due to antibody

dependent complexes (Rweyemamu et al, 1974), or hypersensitivity (Plowright, 1968), or virus induced cell destruction (Selman et al, 1974, Liggit and De Martini 1980b), or virus induced lymphoid transformation (Hunt and Billups 1979) or immune tolerance (Mushi and Rurangirwa 1981c). These theories however cannot adequately account for the T cell hyperplasia that occurs before cell destruction and death.

The hypothesis that best satisfies the clinico pathological picture and the final outcome of the disease is the one proposed by Reid et al (1985). They propose that the virus induced deregulation of lymphocytes is the fundamental mechanism which leads to the production of large granular lymphocytes with natural killer characteristics. They suggest that the target cell for the virus is the large granular lymphocyte and that such infected cells produce lymphokine or interleukins which leads to the T cell proliferation that is seen. The suggestion is supported by the generation of lymphoblastoid cell lines with the phenotypic and functional characteristics of large granular lymphocytes from cattle and deer with OHV-2 induced MCF and rabbits affected with MCF induced by either OHV-2 or AHV-1 (section 1.3.1.2). The tissue destruction may therefore be a direct result of the large granular lymphocytes with natural killer activity affecting tissues *in vivo*. Such granular lymphocytes have been shown to transmit disease to cattle, deer and rabbits without showing detectable viral antigens indicating that these cells are latently infected (Reid et al, 1985). These cells have also been shown to possess T cell surface markers (Burrells and Reid, 1991). Further evidence of the involvement of these cells in the pathogenesis of MCF has been reported using surface markers to analyse cells found in infected tissues (Ellis et al, 1992, Nakajima et al, 1992).

Studies on the pathogenesis of MCF have mainly been hampered by the inability to detect viral antigens in infected cells or tissues and the inability to culture OHV-2. Molecular studies on AHV-1 genome are discussed under genome organisation (section 1.3.4). The complete sequence and organisation of the virulent AHV-1 genome has now been published (Ensser et al, 1997). This has opened an opportunity for elucidation of the mechanisms involved in the pathogenesis of MCF using molecular and immunological techniques.

1. 2. 4 Immunity

1. 2. 4. 1 Response to infection

Various tests have been developed to detect and assay antibody to AHV-1 and OHV-2 (Plowright, 1967, Mushi and Plowright, 1979, Rossiter , 1981, 1982b, Li et al, 1995a). Neutralising antibodies have been found in all free living wildebeest examined and in a few cattle that recover from MCF (Plowright 1968, 1965) but only in 25 -70 % of experimentally infected cattle that develop the disease. The indirect immunofluorescence test has been the most useful test for routine use in serological studies and the test has been used to show that the majority of sheep in the UK and elsewhere have antibody which reacts with AHV-1 (Rossiter et al, 1980b). The drawback with this test is that it is only 90% specific probably due to cross reactions with BHV-2 and BHV-4 (Rossiter et al, 1989).

Wildebeest calves become infected *in utero* or soon after birth and such calves

continue to shed virus until they are 3 months old (Mushi et al, 1980a,b). Neutralising antibody initially derived from colostrum followed by seroconversion is usually detectable in calves. Infection generally occurs in the presence of maternal antibody (Plowright, 1967). Secretory IgA has also been detected in nasal secretions of these calves and this may be important in limiting shedding of virus in the wildebeest calves (Rurangirwa et al, 1982).

The antibody response following experimental infection of cattle and rabbits infected with AHV-1 has been studied by Rossiter (1982b). He showed that the immunofluorescence type of antibody appears in cattle and rabbits 6-7 days before pyrexia and that the titres continue to rise until death. Neutralising antibodies are detectable shortly before fever in the majority of rabbits but no antibody was detected in cattle up to three days after pyrexia. Rossiter (1982b) used the immunoperoxidase test to show that infected rabbits develop IgG and IgM class antibodies although the IgG antibody was present in higher titre than the IgM.

Studies have been carried out to elucidate the viral structural polypeptides which sera from cattle, sheep and wildebeest recognise (Herring et al, 1989). Wildebeest sera were shown to react consistently with 6 polypeptides but sheep and bovine sera from clinical cases of MCF reacted with only a subset of that recognised by wildebeest sera. Cattle sera reacted with 3 of the 6 polypeptides and the authors suggested that this is probably due to low level of replication of the virus or due to incomplete expression of the full genetic repertoire of the virus in cattle.

Cattle that recover from the experimental AHV-1 form of the disease are immune as they fail to succumb to subsequent virulent virus challenge. In these animals, the virus persists in a latent form, as offspring of a recovered dam developed MCF soon after birth (Plowright et al, 1972) and recrudescence of virus has been induced in an

apparently recovered animal following treatment with dexamethasone (Heuschele et al, 1984).

1. 2. 4. 2 Response to vaccination

Several attempts have been made to immunise cattle and rabbits using different immunogens and vaccination regimens with varying results. Reid and Rowe (1973) attempted to immunise cattle with a cell free high passage AHV-2. Two out of three cattle resisted challenge with homologous virulent cell associated virus but succumbed to challenge with virulent AHV-1. Attempts have also been made to immunise cattle with live or formalin inactivated virus preparations with adjuvant (Plowright et al, 1975). Though these cattle had high and persistent humoral antibody responses they were not protected from virulent challenge. Edington and Plowright (1980) showed that rabbits could be protected against cell free virulent virus but not against cell associated AHV-1 after inoculation with inactivated vaccine. In an elaborate experiment involving a regimen of three inoculations with virus inactivated by ultrasonication or acetylethylenediamine, Russell (1980) was able to protect 4/6 rabbits from virulent AHV-1. Rossiter (1982a) protected 2/4 immunised rabbits using a vaccination regimen which entailed 3 inoculations with formaldehyde or glutaraldehyde inactivated infected cells. Though these results indicate that vaccination against MCF is possible they are of limited practical value in the vaccination of cattle.

Thus there is no vaccine available for cattle or other susceptible animals. Furthermore the ability of AHV-1 to maintain latency in cattle that recover after

clinical MCF (Plowright et al, 1972, Rweyemamu et al, 1976) is of prime consideration in the development of vaccines against AHV-1 and OHV-2.

1. 2. 5 Diagnosis

Tentative diagnosis is possible from the presenting clinical-pathological picture especially if there is a history of contact of the sick animals with either of the natural hosts. The disease however may be confused with various other diseases. These include; theileriosis, rinderpest, Ibaraki, or Jembrana diseases in Africa and Asia or with mucosal disease or infectious bovine rhinotracheitis world-wide (Plowright, 1986).

Virus isolation in cell culture is confirmatory but this is only useful with infections with AHV-1 since OHV-2 has not been isolated in culture. With AHV-1 induced MCF virus may be isolated in bovine thyroid, turbinate or testes cell cultures although the former are the most satisfactory using peripheral blood lymphocytes or lymphocyte cell suspensions from affected tissues (Plowright et al, 1960). Typical herpesvirus syncytia normally occur 12-20 days after inoculation of cultures and such cells may be stained using immunohistochemical reagents to identify AHV-1 specific antigens (Ferris et al, 1976, Rossiter, 1981).

The molecular cloning and sequencing of the AHV-1 genome has facilitated the development of a PCR as an aid in the diagnosis of MCF. The PCR for detection of AHV-1 DNA was developed by Hsu et al, (1990b) and Michel, (1993) whereas the technique for detection of OHV-2 has been developed by Baxter et al, (1993). This test is invaluable in the diagnosis of MCF caused by OHV-2 and it has been used to detect OHV-2 in colostrum of lactating sheep (Li et al, 1998) and also in normal

sheep and infected cattle (Wiyono et al, 1994, Baxter et al, 1997)

Various serological tests are available for diagnosis of MCF. These include neutralisation test (Plowright, 1967, Mushi et al, 1979), indirect immunofluorescence (Rossiter, 1980b), and recently a competitive ELISA (Li et al, 1994a) has been described. These tests have limited use in diagnosis as not all affected animals develop antibody, and AHV-1 and OHV-2 antibody have been shown to cross-react with BHV-4 (Rossiter , 1980a,b, Rossiter et al, 1989).

1. 3 Etiology

The transmission of disease with whole blood from cattle affected with the wildebeest form of MCF to susceptible cattle proved that the disease was caused by a cell associated pathogenic agent (Daubney and Hudson 1936). The name malignant catarrhal fever derives from the clinico-pathologic features seen, though epidemiologic studies indicated that the disease could be acquired by infection from two different sources, i. e. the wildebeest in Africa (shown in figure 1.3) and sheep in Europe.

A virus that caused MCF was first isolated in 1959 (Plowright et al, 1960) from five out of 35 blue wildebeest (*Connochaetes taurinus*). The virus was shown to cause MCF in cattle following inoculation of the virus. Studies on the cytopathology and electron microscopy of the virus in cell cultures showed that the virus was closely related to the human herpesviruses. The virus was therefore included in the herpesvirus group (Plowright, 1965). The virus is now designated AHV-1 as its natural host is the wildebeest which belongs to the subfamily Alcelaphinae of the family Bovidae (Roizman et al, 1981).

Viruses have also been isolated by cultivation of primary thyroid cultures from hartebeest (*Alcelaphus buselaphus*) (Reid and Rowe, 1973) and also from topi (*Damaliscus korrigum)* (Mushi et al, 1981b). The former isolate was shown to be antigenically related to AHV-1 and has been designated Alcelaphine herpesvirus -2 (AHV-2) (Roizman et al, 1992). Another virus was isolated by co-cultivation of buffy coats from a roan antelope (*Hippotragus equinus*) showing no signs reminiscent of MCF (Reid and Bridgen, 1991). This virus was shown to induce clinical MCF following injection into rabbits and to be related to AHV-1 using antigenic and DNA cross-hybridisition methods. This virus is now designated Hippotragine herpesvirus-1 (Hip HV-1) (Reid and Bridgen, 1991, Roizman et al, 1992).

The virus that causes the sheep associated MCF has been designated OHV-2 (Roizman 1992). Although this virus has not been isolated in culture epidemiological and molecular evidence implicate sheep as the source of infection for susceptible hosts (Rossiter 1980b, Herring et al, 1989, Baxter et al, 1993, 1997, Li et al, 1994a). These viruses are now classified within the family Herpesviridae (Roizman 1992). The remainder of this section describes the properties of AHV-1 but reference will be made to OHV-2 where appropriate.

1. 3. 1 *In vitro* culture characteristics

1. 3. 1. 1 Cytopathology

AHV-1 causes typical cytopathic effects (cpe) characterised by syncytia and multinucleated cells following co-cultivation of lymphocytes from normal or infected

cattle with bovine thyroid cell cultures (Plowright et al, 1960, 1963). The virus is strictly cell associated and infectivity cannot be demonstrated in cell culture fluids of these infected cells. Such infected cultures show Cowdry type A inclusions which become increasingly basophilic. AHV-1 antigens can be demonstrated by immunofluorescence 24hr post infection although cpe normally occur 3-22 days after infection (Ferris et al, 1976, Wibberley , 1976)

Plowright (1963) developed a strain of AHV-1 recovered from wildebeest by *in vitro* passage to a stage where it could be passaged by inoculation of cell-free fluids. Serial passage in culture was also shown to result in increasing presence of cpe. characterised by cell rounding (Plowright , 1968). An isolate of AHV-1, designated WC11(Plowright, 1965) was shown to become cell free but still retained virulence for cattle after 42 passages in calf kidney cultures (Plowright, 1968). After a further 52-55 passages in calf kidney cells the virus was shown to lose virulence for cattle but the same virus retained virulence for 2/3 cattle following serial passages in calf thyroid cells. A virulent clone of strain C500 (Plowright et al, 1975) which was cell associated was shown to become attenuated after 30 passages in bovine turbinate cells (Handley et al, 1995). Attenuation was also accompanied by production of increasingly cell free virus. There is no clear relationship between loss of virulence and passage level.

Electron microscopic studies on infected cells reveal margination of chromatin and absence of the nucleoli and presence of intranuclear, cytoplasmic and extracellular herpes-like virions (Plowright, et al, 1963, Castro et al, 1981, 1985). The extracellular particles are composed of enveloped and non-enveloped virions ranging in size from 140 to 220nm with an inner capsid core of 100nm (Plowright et al, 1963, Castro et al, 1982). Within the infected cell, the virions are icosahedral, develop in

the nucleus and acquire one or more envelopes (Castro et al, 1982). The morphology of AHV-1 has been confirmed using purified virus preparations (Seal et al, 1988).

1. 3. 1. 2 Cell immortalisation

A cell line with characteristics of large granular lymphocytes following cocultivation of lymph node and spleen suspensions from a rabbit experimentally infected with OHV-2 with feeder cell cultures was described by Reid et al, (1983). These cells were dependent on feeder cultures and, or interleukin 2 (IL-2) but became independent of these substrates after prolonged subculturing. Similar cells have subsequently been cultured from a wide range of tissues derived from animals affected with OHV-2 induced MCF (Reid et al, 1989). These immortalised cells have been shown to be distinctly T-lymphoblastoid as they all possess at least one T-cell surface marker, CD2 (Burrells and Reid, 1991).

Similar cells are also readily produced from AHV-1 infected tissues from experimentally infected rabbits (Reid, personal communication). Furthermore, a cell line derived from AHV-1 infected rabbit cells has been shown to possess some characteristics of lymphokine activated killer cells (Wilkinson et al, 1992). Immortalised cells derived from rabbit, deer and cattle infected with OHV-2 have been shown to be virulent for rabbit without any evidence of virus particles or antigens (Reid et al, 1983, 1989).

Hybridisation experiments using OHV-2 infected cells have shown that these immortalised cells possess DNA homologous with AHV-1 DNA. Handley (1993) extracted OHV-2 DNA from immortalised T lymphocytes infected with OHV-2 and performed a PCR using primers which amplify sequences of AHV-1 and. found that

in the OHV-2 DNA there was a sequence, homologous to the AHV-1 DNA, which represents part of the peptide 1 of AHV-1 (discussed in section 1.3.4). So far however, there is no evidence as to which viral transcripts are expressed by these cells.

1. 3. 2 Nomenclature and classification

The herpesvirus group of the International Committee for the Taxonomy of viruses recommended a binomial system of nomenclature based on three main biological determinants; (1) the first name should reflect the family of the natural host of the virus (when the name should end in -id) or subfamily (when the name should end in -ine) (2) the second name should be herpesvirus and (3) different herpesviruses with the same primary host should be given sequential arabic numbers (Roizman et al, 1981). The virus of wildebeest is therefore called Alcelaphine herpesvirus-1 to denote the subfamily of the natural host (Reid et al, 1975, Roizman et al, 1981).

Previously the biological criteria used to classify herpesviruses was based on simple but practical measurements and observations (Roizman et al, 1981). The herpesviruses were divided into three subfamilies; Alphaherpesviruses, Betaherpesviruses, and Gammaherpesviruses to which the viruses of MCF belong .

A method of classification based on genomic organisation of the herpesviruses has also been used . AHV-1 has been classified as a member of group B (figure 1.4) on the basis of its genomic structure (Bridgen et al, 1989).

1. 3. 3 Gammaherpesviruses

All members of the gammaherpesvirus subfamily replicate in lymphoblastoid cells, and some also cause lytic infections in fibroblastic cells *in vitro*. The viruses in this group are specific for either T or B lymphocytes. In the lymphocyte the infection is either prelytic or lytic but without production of infectious progeny (Roizman et al, 1981)

The subfamily is at present divided into 2 genera, namely the Lymphocryptovirus, members of which are typically B-lymphotropic, and Rhadinovirus, members of which are typically isolated from T-lymphocytes. A list of animal gammaherpesviruses is presented in table 1.1.

AHV-1 is classified as a gammaherpesvirus of the Rhadinovirus group. Prototype viruses in this group are; Ateline herpesvirus-2, Saimirine herpesvirus-2 (referred to as Herpesvirus saimiri, HVS), Bovine herpesvirus-4 and Murid herpesvirus-4, (commonly referred to as mouse herpesvirus 68), as defined by Roizman et al, 1992

Herpesviruses are also classified according to their genomic arrangements (Roizman et al, 1992, presented here in Figure 1.4). Herpesvirus DNA is characterised by a long stretch of unique DNA with low G+C ratio flanked by tandem repeats with high G+C ratio. AHV-1 has been included within the B group of the gammaherpesviruses (Seal et al, 1989, Bridgen and Reid, 1991). The viral DNA in this group of viruses is arranged in such a way that the terminal sequence is directly repeated several times at both termini (Roizman et al, 1992).

Figure 1.3; Group of adult blue wildebeest in the Masai Mara

TABLE 1.1; Animal viruses comprising the subfamily Gammaherpesviruses

Designation	Common name(synonym)	G+C*	Group[a]	Size [b]	Reference
Human HV 4	Epstein-Barr virus	60	C	172	Baer et al, (1984), Epstein et al, (1965), Kief and Liebowitz, (1990)
Human HV 8	Karposis sarcoma associated virus	-	-	-	Chang et al, (1994), Ambroziak, et al (1995)
Cercopithecine HV 12	HV papio, baboon HV	-	C	170	Falk et al, (1976), Heller et al, (1982)
Equine HV 2		57.7%	B	196	Telford et al, (1993), Wharton et al, 1981)
Equine HV 5		57.7%	C	179	Telford, et al, (1993), Plummer et al, (1973), Ebeling, et al, (1983)
Cercopithecine HV 15	Rhesus EBV-like HV				Rangan et al, ((1986)
Ateline HV 2	HV ateles	48	B	135	Deinhardt et al, (1973), Fleckenstein et al, (1978), Fleckenstein and Desroisers, (1982), Melendez et al, (1972)
Pongine HV 1	Chimpazee HV, pan HV	-	C	170	Heller et al, (1981), Landon et al, (1968)
Pongine HV 2	Orangutan HV				Rasheed et al, (1977)
Pongine HV 3	Gorilla HV				Neubauer et al, (1979)
Saimirine HV 2	Squirel monkey HV, HV saimiri	46	B	155	Bornkamm et al, (1976), Cox et al, (1980), Trimble et al, (1991)
Bovine HV 4	Movar HV	50	B	145	Bartha, et al, (1966), Bublot, et al, (1990) , Thiry et al, (1989), Todd and Storz, (1983).

Ovine HV 2	Sheep associated malignant catarrhal fever of cattle HV				Bridgen and Reid, (1991)
Alcelaphine HV 1	wildebeest HV, Malignant catarrhal fever HV of European cattle	61	B	160	Bridgen and Reid, (1991), Bridgen et al, (1989), Plowright et al, 1960), Plowright et al, (1965a,b).
Alcelaphine HV 2	hartebeest HV	-	B		Reid and Rowe, (1973).
Leporid HV 1	Cotton tail HV, HV sylvilagus	33	B	145	Goodheart and Plummer (1974), Medveczky et al, (1989), Nesburn, (1969).
Murid HV 4	Mouse HV strain 68		B	135	Blaskovic et al, (1980), Efstathiou et al, (1990), Rajcani et al, (1985)
Marmodid HV 1	Woodchuck HV, HV marmota	-	B	135	Gilles and Ogstron, (1991), Schechter et al, (1988).
Porcine lymphotropic HV 1	Porcine HV				Ehlers et al, 1999
Porcine lymphotropic HV 2	Porcine HV				Ehlers et al, 1999

[*] = % mole [a] = Group based on genomic organisation [b] = genome size in kbp HV=Herpesvirus

B= Rhadinovirus C= Lymphocryptovirus

Key to figure 1.4

LTR and RTR - left and right terminal repeat in group A

R1 to R4 - internal repeats of group C

IR and TR - internal and terminal repeats of group D

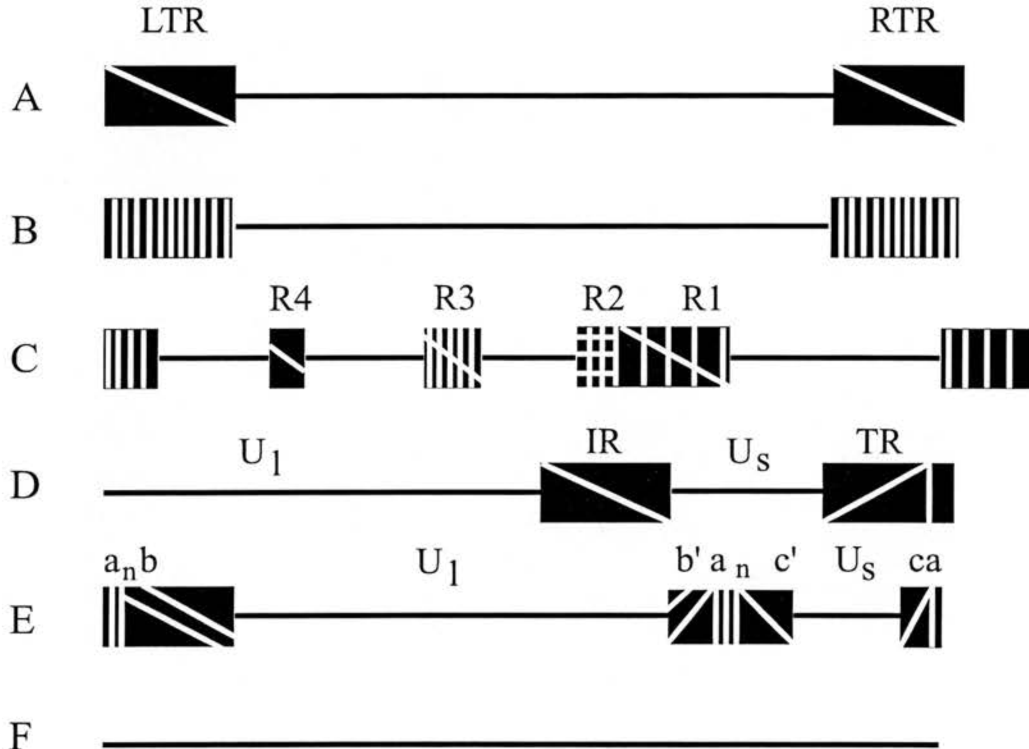
a and b - repeats in group E

n - number of repeated sequences

UL and US - unique long and short sequences in group E

Figure 1.4. Classification of Herpesviruses on the basis of genomic arrangement

(Taken from Roizman et al, 1992)



A. Channel catfish herpesvirus

B. Herpesvirus Saimiri

C. Epstein Barr virus

D. Varicella Zoster virus

E. Herpes Simplex virus

F. Tree Shrew herpesvirus

1. 3. 4 Genomic organisation

The first characterisation of the AHV-1 genome was performed by Bridgen et al, (1989) using DNA of the attenuated WC11 strain (Plowright et al, 1965a,b) and the virulent strain C500 (Plowright et al, 1975). The genome of the attenuated strain was found to comprise of a region of unique DNA of approximately 130Kbp with a G+C content of 50%, and approximately 30Kbp of additional direct repeats with a G+C of 72%. Although the virulent strain of AHV-1 DNA had a similar restriction profile to WC11, the repeat sequence was shown to be 1050bp in length whereas that of the attenuated strain was 950bp. These repeats were shown to be terminal in location, (Seal et al, 1989, Bridgen and Reid, 1991), and it was suggested that this virus be included in the B group of the gammaherpesviruses which includes Herpesvirus saimiri (Albrecht et al, 1992) and equine herpesvirus-2 (Telford et al, 1995).

Further restriction maps have been produced to determine the genetic relationships between AHV-1 and other viruses isolated from cattle and also with related viruses isolated from the Alcelaphinae subfamily, (Seal et al, 1989, Shih et al, 1989). Shih et al, (1989) compared genomes of herpesviruses associated with MCF and assigned the viruses into two distinct groups; one group comprising isolates from blue wildebeest (*Connochaetes taurinus taurinus*), Formosan sika deer (*Cervus nippon taiouanus*) and Siberian ibex (*Capra ibex sibirica*) and the other group comprising of isolates from greater kudu (*Tragelaphus strepsiceros*), cape hartebeest (*Alcelaphus, buselaphus*), white tailed wildebeest (*Connochates gnu*), white bearded wildebeest (*Connochaetes taurinus albojubatus*) and the cattle isolate C500. Seal et al, (1989) compared the wildebeest isolates with isolates from the topi and hartebeest and found

that these were two isolates, each distinct from the other and different from the cattle isolates. These viruses are now designated AHV-1 and AHV-2 (Roizman et al, 1992).

Studies have been carried out to sequence the L-DNA adjacent the terminal repeats (Handley, 1993, Handley et al, 1995). The main objective was to determine whether there were sequences homologous to the transforming ORFs identified in another gammaherpesvirus, the herpesvirus saimiri (Beisinger et al, 1990). This work was carried out by comparing the virulent isolate, C500 (CA) (Plowright et al, 1975), the attenuated isolate, WC11 (Plowright et al, 1960) and a high passage avirulent cell free C500 (CFA). Clones of DNA from these virus isolates derived from the region adjacent to the terminal repeats were used to prepare restriction maps and subclones to facilitate sequencing.

The results showed that the virulent C500 contains two copies of a sequence of approximately 2Kbp adjacent to the terminal repeats at the left end of the molecule. In the attenuated virus there were also two copies of the 2Kbp sequence but these were located at both ends of the molecule, adjacent to the terminal repeats. Analysis of amino acid translations of these sequences revealed 9 hypothetical polypeptides.

One open reading frame (ORF), designated polypeptide 5, was altered on attenuation such that the 3' end of the molecule was lost (Handley, 1993). However comparison of this alteration with the deletion that occurs in another B group rhadinovirus led to the changes being orientated towards the left end of the genome (Handley et al, 1995) This ORF, of 496bp, was compared to all other sequences in the databases and no significant homology was observed. Analysis showed that this ORF would generate a polypeptide of 140 amino acids and would contain a region of 15-20 hydrophobic residues. The polypeptide would also contain a region of 100-130 aminoacids that

was highly antigenic. Comparison of the location of this reading frame and other B-group rhadinoviruses, suggested that this ORF was similar to the Saimiri transforming proteins, STP-A11 and STP-C488 of HVS subgroups A11 and C488 respectively (Murphy et al, 1989, Biesinger et al, 1990, Albrecht et al, 1992). Passage of HVS in culture results in the deletion of the STP-A11 gene that is associated with transformation (Murphy et al, 1989, Biesinger et al, 1990). The authors therefore suggested that polypeptide 5 in AHV-1 may be responsible for virulence.

Another open reading frame, encoding a hypothetical polypeptide designated polypeptide 1, consisting of 300 amino acids, was common to all isolates. Analysis of this ORF showed structural homology to the Tyrosine kinase interacting protein (TIP) of HVS, (Murphy et al, 1989, Beisinger et al, 1990). The presence of two copies of this reading frame however indicated that this protein may not have a similar functional role (Handley, 1993). The presence of this ORF in the virulent and the attenuated viruses and also in immortalised cells is important in diagnosis and expression studies.

During the course of this work, the complete sequence for virulent virus has been published and the organisation of the AHV-1 genome confirmed using a cell free but virulent derivative of the C500 isolate (Ensser et al, 1997). This is presented in figure 1.5. and table 1.2

Figure 1.5 Organisation of the AHV1 C500 genome (after Ensser et al, 1997).

Putative homologues of the potential ORFs are described in table 1.2.

ORFs have been coloured to reflect the conservation of genes between the α, β and γ subfamilies of the Herpesviridae as indicated below.

ORFs specific to AHV1

ORFs specific to the γ subfamily

ORFs specific to the $\beta + \gamma$ subfamilies

ORFs specific to the $\alpha + \gamma$ subfamilies

ORFs common to all herpesviruses

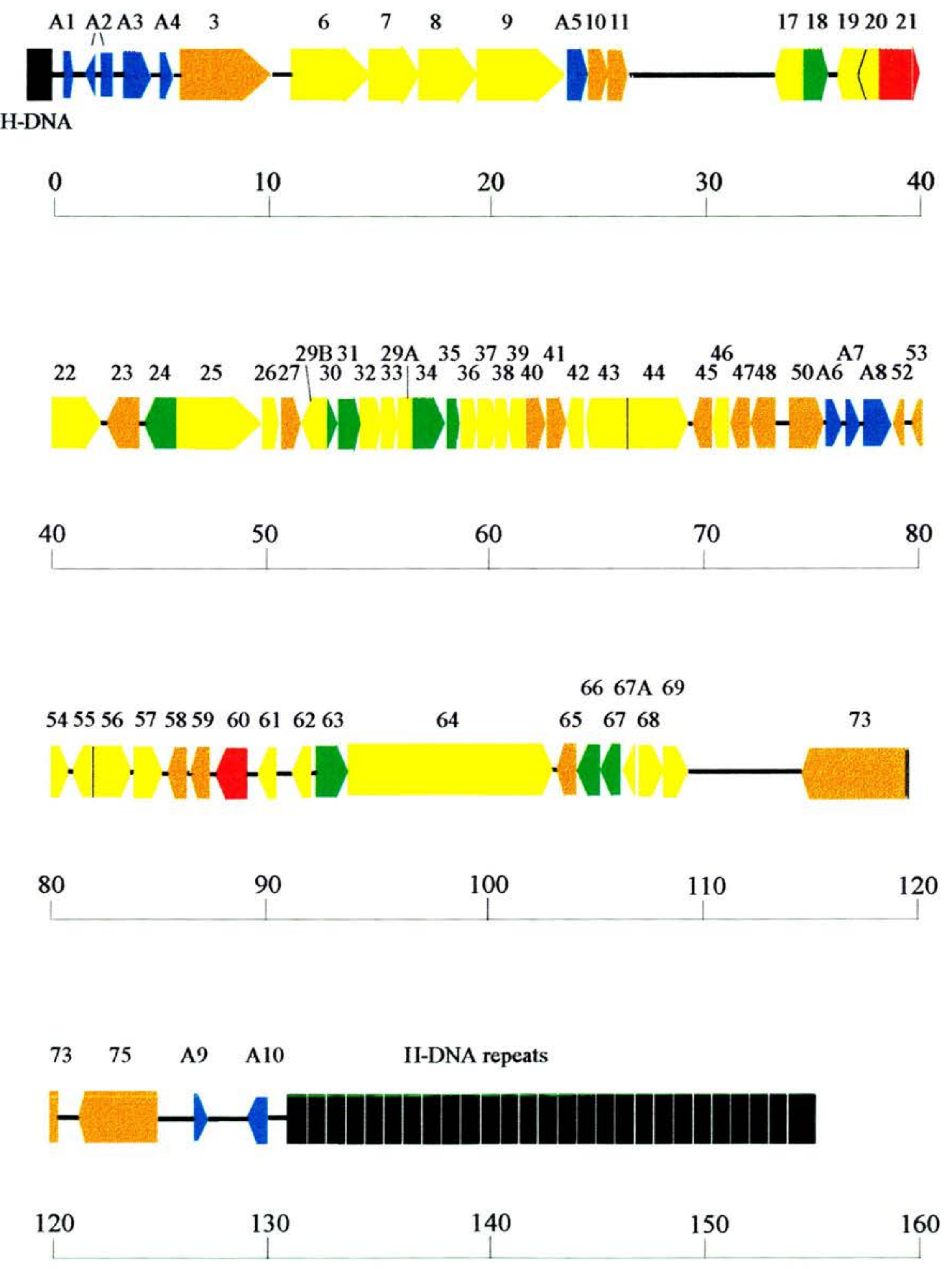

A1 A2 A3 A4 3 6 7 8 9 A5 10 11 17 18 19 20 21
H-DNA
0 10 20 30 40
22 23 24 25 26 27 29B 31 30 32 33 29A 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 50 A6 A7 A8 52 53
40 50 60 70 80
54 55 56 57 58 59 60 61 62 63 64 65 66 67A 67 68 69 73
80 90 100 110 120
73 75 A9 A10 II-DNA repeats
120 130 140 150 160
kbp

TABLE 1.2. AHV-1 Open reading frames (ORF, after Ensser et al, 1997).

ORF	+/-	Start*	Stop*	aa[a]	Description[b]
A1	+	918	1211	97	
A2	-	2230	1796	144	Exon 2 (144aa++)
A2	-	2477	2313	55	Exon 1(55aa)
A3	+	3492	5453	653	Semaphorin homolog
A4	+	5732	6097	121	Signal peptide
3	+	6333	10442	1369	Viral protein, FGARAT
6	+	11260	14643	1127	Major ssDNA binding protein
7	+	14725	16767	680	Processsing and transport protein
8	+	16751	19315	854	Glycoprotein B
9	+	19428	22508	1026	DNA polymerase
A5	+	22784	23692	302	Probable GPCR membrane protein
10	+	23774	24988	404	Raji LF1 after reconstruction of deletion site
11	+	25002	26222	406	
17	-	35377	33808	524	Protease and capsid protein
18	+	35337	36164	275	
19	-	37808	36138	556	Virion tegument protein
20	-	38346	37594	250	Fusion protein
21	+	38345	40030	561	Thymidine kinase
22	+	40059	42260	733	Glycoprotein H, signal peptide
23	-	43448	42253	401	
24	-	45653	43416	745	
25	+	45655	49767	1370	Major matrix protein
26	+	49818	50738	306	Capsid protein vp23
27	+	50745	51623	292	

29	-			686	Late spliced gene, DNA packaging, possible terminase
29b	-	52824	51682	380	Late spliced gene,DNA packaging protein, possible terminase, exon 2
30	+	52840	53097	85	
31	+	53016	53693	225	
32	+	53642	55066	474	
33	+	55056	56063	335	
29a	-	56970	56053	306	Late spliced gene, DNA packaging protein, possible terminase, exon 1
34	+	56969	58000	343	
35	+	57957	58415	152	
36	+	58318	59682	454	Phosphotransferase, possible tyrosine kinase
37	+	59615	61072	485	Alkaline exonuclease
38	+	61027	61206	59	Myristylated in HSV
39	-	62374	61250	374	Integral membrane protein, glycoprotein M (CMV)
40	+	62481	63917	478	Helicase-primase complex
41	+	63996	64523	175	Helicase-primase complex
42	-	65293	64520	257	
43	-	66950	65277	557	Minor capsid protein, virion protein
44	+	66940	69291	783	Helicase
45	-	70055	69348	235	
46	-	70846	70058	252	Uracil-DNA glycosylase
47	-	71285	70779	168	Glycoprotein L (CMV)
48	-	72662	71402	419	
50				619	Trancriptional control, Rta homolog

50x1	+	72825	72901	26	Rta homolog, exon 1
50x2	+	73121	74903	593	RtA homolog, exon 2
A6	+	75226	75858	210	
A7	-	76569	77300	243	Signal peptide
A8	+	77293	79344	683	Putative glyoprotein; Similarity to EBV receptor
52	-	79775	79398	125	
53	-	80165	79854	103	Signal peptide
54	-	80228	81124	298	dUTPase
55	-	81824	81162	220	
56	-	81823	84336	837	Helicase-primase complex.
57a		84462	84513	16,33	Transcriptional control exon 1 homolog
57b	-	84604	85863	418,67	Transcriptional control exon 2 homolog
57				435	Transcriptional control IE52 homolog
58	-	87077	86022	351	
59	-	88319	87084	411	Processivity factor/subunit of DNA polymerase
60	-	89347	88430	305	Ribonucleotide reductase (small unit)
61	-	91720	89378	780	Ribonucleotide reductase (large unit)
62	-	92740	91736	334	Probable capsid assembly and DNA maturation protein
63	-	92739	95597	952	Tegument protein
64	-	95612	103432	2606	Large tegument protein
65	-	104231	103473	252	Capsid protein
66	-	105617	104304	437	
67	-	106312	105521	263	Virion tegument protein
67A	-	106573	106319	84	

68	-	106760	108166	468	Probable major envelope glycoprotein
69	-	108189	109031	280	
73	-	120278	116376	1300	Glycine rich, repetitive region
75	-	125243	121296	1315	Virion protein, FGARAT
A9	-	125771	126277	168	Bcl2 homolog
A10	-	130229	128811	472	Possible glycoprotein

+/- indicates whether the ORF is on the positive or negative strand of the AHV-1 genome.

* indicates the positions of start and stop codons.

[a] indicates the number of amino acids in the ORF.

[b] Possible functions of the AHV-1 proteins are deduced from the homologous ORFs described in other herpesviruses.

1. 3. 5 Viral Proteins

Analysis of purified virion proteins using PAGE polypeptide profiles of AHV-1 and AHV-2 indicated that these viruses contain at least 36 polypepetides with molecular weights ranging from 12kDa to 275kDa (Seal et al, 1989). Further characterisation of cell free AHV-1 polypeptides using polyclonal serum in an immunoprecipitation experiment identified a complex of polypeptides with molecular weights, 115, 110, 105, 78, and 45kDa. (Li et al, 1995a). The 45kDa moiety however was the only polypeptide recognised by monoclonal antibodies raised to a conserved epitope of AHV-1 isolates from the zoological collections in the USA. The polypeptide profile observed was similar to that observed on viral envelope proteins characterised by Adams and Hutt-Fletcher (1990).

Studies have been performed to screen cDNA libraries derived from mRNA transcribed in AHV-1 infected cell cultures (Lahijani et al, 1994). A cDNA clone, of 833bp was identified which coded an immunoreactive protein of 15kDa. This sequence showed greater than 40% DNA homology to the B group gammaherpesviruses HVS, EBV and the cottontail tamarin virus (*Saguinus oedipus oedipus*). This clone was shown to hybridise to a 4.4kbp AHV-1 DNA fragment in a Hind III digest. This Hind III fragment has been shown to be derived from the unique region of the AHV-1 near to the terminal repeats (Bridgen, 1990).

1.3.6 Replication of gammaherpesviruses

Studies on replication of gammaherpesviruses have mainly been conducted in EBV (

Kieff and Liebowitz, 1990). EBV is a gammaherpesvirus that infects humans and may cause various forms of cancer in humans (Kieff and Liebowitz, 1990). The virus can latently infect and immortalise B lymphocytes (Pope, 1968, Nilsson et al, 1971) but a proportion of these latently infected cells do replicate virus with production of mature virus particles. In latently infected cells only a few viral genes are expressed and productive virus replication does not occur (Dambaugh et al, 1984). However some of these cells have been shown to spontaneously allow lytic replication of EBV with production of mature virus. Induction of the lytic phase of EBV replication can also be induced with phorbol-esters e.g. TPA G with release of mature virus particles (Zur Hansen et al, 1978).

T cells can be derived from rabbits experimentally infected with AHV-1 although such latently infected cells do not enter a lytic phase of replication with productive virus replication (Reid-personal communication). Studies have been conducted to elucidate the mediators and mechanisms involved in the maintenance of latency and the factors involved in the switch of these cells into the productive cycle of virus replication in herpesviruses.

In EBV, viral DNA circularises following entry into peripheral blood lymphocytes and the episomal DNA can be detected in the nuclei of these cells 24 hr after infection (Alfieri et al, 1991). Large cytotoxic lymphocytes (LCL) stably infected with EBV contain multiple copies of circularised EBV viral DNA (Adams and Lindahl, 1975) and also in LCL immortalised by AHV-1 (Bridgen et al, 1989). The initial amplification of the EBV episomal virus occurs at the start of latency during the S phase of the cell cycle as only single copies of viral DNA are present before the S phase (Sudgen et al, 1979, Alfieri et al, 1991). Replication in latency is bidirectional and originates from a site in the episomal DNA called oriP. Replication

is dependent on expression of viral proteins, designated EBNA1, EBNA2 and LMP1 (Alfieri et al, 1991) and is facilitated by cellular DNA polymerase. There are however no homologues for EBNAs or LMP1 in rhadinoviruses (Kieff and Liebowitz 1996, Albrecht et al, 1992) indicating that viruses in this group of gammaherpesviruses probably utilise different effector proteins. Viral DNA replication during latency is synchronised with the cell cycle as viral DNA copy number is maintained in daughter cells.

Establishment of latency is also accompanied by methylation of viral DNA and treatment of cells with chemical agents that reduce DNA methylation increases the level of spontaneous activation to the lytic phase (Saemundsen et al, 1980, 1983, Ben-Sasson and Klien, 1981)

Induction of the lytic replication in EBV is accompanied by increase in copy number of circular viral DNA which is dependent on viral DNA polymerase (Shaw 1985). Replication is also dependent on transcription of two immediate early proteins, encoded by BZLF2 and BRLF1 (Chevallier-Greco et al, 1986, Ragoczy et al, 1998) Replication is initiated from either of two regions on the viral genome at any one time, called oriLyt to yield head to tail concatamere (Hammerschimdt and Sudgen, 1988). During the lytic phase of EBV replication, many copies of viral DNA are produced showing that this type of replication is independent of cellular DNA replication.

Probably the terminal repeats in AHV-1 generated DNA during lytic replication serves as signals for cleavage and packaging as observed in alphaherpesviruses (Spaete and Flenkel, 1982).

Infection of wildebeest is accompanied by lytic cycle of AHV-1 replication probably in the nasal and upper respiratory mucosae and shedding of mature virus before

establishment of latency in peripheral blood lymphocytes (Plowright 1986). In cattle however, there is no lytic replication of virus and the infection is essentially latent since no cell free virus is shed. It is therefore probable that the lytic phase of virus replication is a prerequisite to the development of immunity in AHV-1. In the natural host the absence of a clinical response following infection may be due to presence of cellular factors that induce lytic virus replication or may be a consequence of immunological response to viral proteins that are expressed during the lytic cycle of AHV-1 replication.

1.4 AIMS OF THIS PROPOSAL

During the initiation of this work, the aim was to clone, express and determine the role played by the putative proteins of virulence described by Handley (1993), Handley et al, (1995) in MCF. Subsequently, the complete sequence of the virulent AHV-1 genome showed that the ORFs were only part of longer ORFs and that the ORF for both proteins were located in the middle of the genome (Ensser et al, 1997). Since the virus used was a 1975 isolate which has undergone various passages in tissue culture, the genotype of this virus may have been altered.

The second aim of this proposal was therefore to obtain fresh isolates of AHV-1 and compare viral DNA derived from these isolates with AHV-1 DNA derived from low and high passage C500 isolate in tissue culture monolayers as well as viral DNA obtained from the attenuated WC11 isolate to determine the extent to which some of the putative genes of virulence are conserved.

Isolation and characterisation of new isolates of AHV-1 was conducted at the NVRC, Muguga, Kenya. All the other experiments were conducted at MRI.

CHAPTER 2

MATERIALS AND METHODS

2. 1 Suppliers

Analar grade chemicals were purchased from BDH, Fisons, Sigma, Boehringer Mannheim, Invitrogen, Qiagen, Amersham or Biorad. All bacteriological media and reagents were purchased from Invitrogen and molecular biology reagents were purchased from Bethesda Research Laboratories, Invitrogen, Qiagen, Amersham or Boehringer Mannheim. Immunological reagents were purchased from Scottish Antibody Production Unit (SAPU), Boehringer Mannheim, Sigma, or Invitrogen. The list of suppliers and addresses are listed (see page xvi)

2. 2 Formulation of general solutions

TE 10 mM Tris-HCl pH 8.0, 1 mM EDTA

TNT 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05%Tween 20

TBE 134 mM Tris base, 44 mM Boric acid, 2.6 mM EDTA (10X stock)

DNA loading dye 10% (w/v) Ficoll, 0.05% (w/v) Xylene cyanol, 0.025% (w/v) bromophenol blue

RNA loading dye 0.75 ml formamide, 0.15 ml 10x MOPS, 0.24 ml formaldehyde, 0.1 ml glycerol, 0.1 ml 10% (w/v) bromophenol blue

PBS 150 mM NaCl, 6 mM K_2HPO_4, 4 mM KH_2PO_4 pH 7.2

20XSSC 3.0 M NaCl, 0.3M Sodium citrate

10X MOPS 0.2 M MOPS, 50 mM Sodium acetate, 1 mM EDTA, pH 7. 0

Ponceau stain 2% Ponceau, 30% Sulfasalicylic acid, 30%Trichloroacetic Acid

2. 3 Virological methods

2.3.1 Cell culture and cultivation of virus

Propagation of large granular lymphocytes was conducted by Dr. H. Reid, MRI as described in Reid and Buxton (1985). Cultivation of virulent virus in bovine turbinate cell monolayer cultures was conducted by Miss I. Pow at MRI. The virulent virus was cell associated or cell free derivatives of the C500 isolate (Plowright et al, 1975)

2.3.2 Preparation of DNA from AHV-1 infected bovine turbinate (BT) cultures

Extraction of viral DNA from BT cultures was conducted at MRI. Cell associated virus was harvested when over 80% of the cells exhibited cytopathic effect (cpe). Since the cpe. resulted in the majority of the monolayer cells forming loosely attached syncytia, these cells were easily incorporated into the supernatant and any residual cells were removed from the flask walls by rocking briefly with sterile glass beads. The culture supernatant was then spun at 3000g for 10 min at room temperature and the cell pellet washed with PBS. The cells were resuspended and lysed in 0.5% (v/v) NP40 in RSB (10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) by gentle homogenisation. Chromatin and cellular debris were removed by centrifugation at 1000g for 10 min at room temperature. The supernatant was layered over 25% sucrose in RSB and the viral nucleocapsids were isolated by centrifugation at 114,000g for 30 min at RT. The resultant pellet was resuspended in TNE and digested with proteinase K (Boehringer Mannheim) at 200μg ml^{-1} in the presence of

1% SDS at $37^{0}C$ for 2hr with gentle agitation. Contaminants were extracted with phenol and the DNA precipitated with ethanol.

2.4 Bacteriological techniques

2.4.1 Strains of *E.coli* used in this study

strain	genotype	reference
JM109	*rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi*-D(*lac*-proAB) F' [*tra*D proAB^{+}*lac*Iq*lac*ΔM15]	Yannitch-Perron et al 1985
G1724	F^{-}, l^{-}, lac^{q}, lacPL8, *amp*C::P_{trp}*c l*	Invitrogen

2. 4. 2 Growth media

TABLE 2.1; media used for culture of *E.coli*

RM Media	16.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM, 8.5 mM NaCl, 18.7mM NH_4Cl, 1mM $MgCl_2$, 0.2 % (w/v) Casamino acids
Induction Media	As RM plus 0.5 % (w/v) Glucose
2x TY	1.6% (w/v) Tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl
LB Broth	1% (w/v) Tryptone, 1% (w/v) yeast extract, 1% (w/v) NaCl
L Agar	LB containing 15 g/l Difco agar
RMG	RM media plus 2% Casamino acids, 1mM $MgCl_2$, 1.5% agar
SOC	2% (w/v) tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 20mM Mg^{2+}, 20mM Glucose, pH 7.0

All media except SOC were sterilised by autoclaving for 15 min at 1.05 Kg cm^{-2}. Ampicillin was added to 100 μgml^{-1} after autoclaving. SOC was sterilised by filtering through 0.2μM millipore filters

2. 4. 3 Preparation of competent bacteria

E. coli were streaked on LB plates and incubated overnight at 37^0 C. A single colony was picked and used to inoculate 100 ml of LB broth. The bacteria were allowed to grow to an OD_{600} of between 0.5 to 0.6 when the cells were transferred to prechilled tubes and incubated on ice for 10 min. The bacteria were then spun down using a Beckman rotor J2-21 at 4000rpm for 10 min. The media was decanted and the resuspended in ice cold 0.1M $CaCl_2$ and then placed on ice for 30 min. The bacteria were pelleted again at 4000rpm for 10 min and then resuspended in a volume of $CaCl_2$ containing 15% (w/v) glycerol corresponding to 1/ 50 of the original volume of medium. The bacteria could then be used directly or stored at -70^0 C.

2. 5 Molecular biology techniques

TABLE 2.2; Cloning vectors used in this study

Vector-	Type	size(kbp)	Markers-	Replicon	Promoters
pGEX	Plasmid	4. 94	Amp*lacZ*	f1	T7
pGEM-T	Plasmid	3.0	amp*lacZ*	f1	T7, SP6
pBluescript ($KS^{+/-}$)	Plasmid	2.96	amp*lacZ*	f1	T3,T7
pGL2	Plasmid	5.0	ampluc^+	ColE1, f1	SV40

TABLE 2.3; Vectors and cloning sites

VECTOR	MULTIPLE CLONING SITES
pBluescript* (KS$^{+/-}$)(Stratagene)	Sac1, Bst I, Not I, Eag I, Xba I, Spe I, Bam H1, Sma I, Pst I, EcoRV, Hind III, Acc I, Sal I, Xho I, Apa I, Dra I, Kpn I
pTRX (Invitrogen)	Kpn I, Sma I, BamH I, Xba I, Acc I, Sal I, Pst I
pGEM-T* (Promega)	Apa I, Aat II, Sph I, BstZ I, Nco I, Sac II, Spe I, Not I, Pst I, Sal I, Nde I, Sac I, BstX I, Nsi I
pGL2 (Promega)	Kpn I, Sac I, Mlu I, Nhe I, Sma I, Xho I, Bgl II

*Successful cloning of insert into these vectors interrupts the β–galactosidase sequences. Competent bacteria transformed with these recombinant plasmids therefore form white colonies instead of blue colonies when grown on agar containing X-gal and IPTG.

2. 5. 2 Restriction endonuclease digestion of DNA

Restriction enzyme digestions were performed to create ligation sites in viral DNA PCR products and plasmids and to confirm the presence of these inserts in the recombinant plasmids. DNA digestions were performed with enzymes at a final concentration of 10units/μg of DNA. The restriction enzymes and the respective buffers were purchased from Boehringer Mannheim and used according to the manufacturer's instructions. Between 100 ng and 200 ng of DNA was used for each reaction and RNase was added when required to a final concentration of 10μg ml^{-1}.

Reaction volume was adjusted to 20 μl and digestions were performed by incubating the reaction mixtures at 37^0C for 1hr.

2. 5. 3 DNA ligation

DNA ligation reaction mixtures with all plasmids used in this study except for pGEM-T were prepared using appropriate amounts of insert DNA and vector DNA (at a vector/insert ratio of 1:5), and 10x ligation buffer (30mM Tris-HCl, pH 7.5/10mM $MgCl_2$/10mM DTT/1mM ATP) (Promega). 1 μl of T4 DNA ligase (Promega) was added and the reaction volumes were adjusted to 20 μl with water. Ligations were performed at 37^0 C for 2 hrs or overnight at 4^0C.

PCR products were also cloned into the pGEM-T vector. The vector system takes advantage of the non-template dependent addition of a single deoxyadenosine to the 3' end of PCR products by thermostable *Taq* which can then be ligated into pGEM-T using the T4 ligase enzyme (Promega). Total reaction volume was made up of 1μl:T4 DNA ligase buffer, 50ng vector DNA, insert DNA (at an insert/ vector ratio of 5:1) and 1 Wiess Unit of T4 DNA ligase (Promega). Tubes were incubated overnight at 4^0C

2. 5. 4 Transformation of competent *E.Coli*

10ng of plasmid were added to 50 μl of competent *E.coli* and the contents gently mixed. The tube was kept on ice for 30 min. The cells were heat shocked at 42°C for 90 sec, chilled immediately on ice for two min and then plated on suitable agar plates. The plates were kept overnight at 30^0C when growing *E.coli* G1724 and 37^0C

when growing JM 109 cells. Transformed *E.coli* were selected by the ability to grow in presence of ampicillin. This method was found to be unsuitable for transforming JM109 cells with pGEM-T recombinant plasmids as the efficiency of transformation was always low. A modified method was therefore used. Bacteria transfected with pGEM-T plasmid were heat shocked for exactly 2 minutes at 42^0C and placed immediately on ice for 2 minutes. 900μl of cold SOC medium was added to the transformation reaction and incubated for 60 minutes at 37^0 C in a shaking incubator (approximately 225rpm). The cells were pelleted by centrifugation at 1000xg for 10 minutes and the cells resuspended in 100μl of SOC. The cells were then plated out on LB or 2xTY plates and incubated at 37^0 C for colonies to grow.

2. 5. 5 Small scale preparation of plasmid DNA

Plasmid DNA was prepared from 10 ml overnight cultures. Bacteria were pelleted by centrifugation in a microfuge at 9000g for 5 min and then resuspended in 0.2 ml of glucose buffer. 0.4 ml of 0.2M NaOH, 1% SDS was added to the contents of the tube, mixed by inversion and the tube placed on ice for 5 min. 0.3 ml of High Salt (3M Potassium acetate, 1.8M formic acid) solution was then added and the contents mixed by inverting the tube. The contents were incubated on ice for 15 minutes and the tube centrifuged for 5 min at 9000g to pellet the cellular debris. 0.8 ml of the supernatant was then mixed with 0.48 ml of isopropanol. DNA was pelleted by spinning the tube at 9000g for 10 min. DNA pellet was washed with 75% ethanol and the pellet resuspended in 50 μl TE.

2. 5. 6. Large scale preparation of plasmid DNA

A single bacterial colony was inoculated into 10 ml of suitable media and grown for a few hours at 37^0 C with shaking. This culture was then used to inoculate 500 ml of media. This bulk culture was grown overnight at 37^0 C with shaking. The bacteria were pelleted by centrifugation at 4000g for 30min at 4^0 C. The bacterial cells were resuspended in 20 ml of glucose buffer (50mM Glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA and 40 ml of 0.2M NaOH with 1%SDS added. The contents were mixed gently and 30 ml of High Salt solution (3M Potassium acetate, 1.8M formic acid) added, and shaken before being placed on ice for 30min. Cellular debris was pelleted by centrifugation at 4000g for 30min and 80 ml of the supernatant added to 48 ml of isopropanol. The tubes were placed on ice for 30min and the DNA pelleted by centrifugation at 4000g for 30min. The pellet was resuspended in 8 ml of T.E, 1.0 ml of 10mg ml^{-1} ethidium bromide solution and 9g CsCl were added. The contents were transfered to "quickseal" centrifuge tubes. The tubes were spun at 40000rpm in a Beckman Ti70 rotor for 36 hrs. The lower band containing plasmid DNA was collected using an 18 gauge needle. Ethidium bromide was removed from the DNA by extracting with water saturated butan-1-ol, and the DNA precipitated by addition of 1 volume of water and 2 volumes of ethanol. The concentration of the plasmid DNA was determined using a spectrophotometer

2.5.7 Preparation of plasmid ssDNA

A single colony known to contain the plasmid of interest was inoculated into 2ml of LB containing VCS-M13 helper phage (Stratagene) at a concentration of 10^7pfu/ml. The bacteria were incubated at 37^0C for 90 minutes in a shaking incubator when

Kanamycin was added to a final concentration of 70μg/ml. The cells were incubated overnight in a shaker at 37^0C. The bacteria were pelleted by centrifugation at 9000g for 10 minutes and 1.2 ml of the supernatant transferred to a microfuge tube containing 300μl of 20%PEG/2.5M NaCl. The contents were mixed and kept at room temperature for 15 minutes. The contents were centrifuged at 9000g for 10 minutes to precipitate the phage. The phage pellet was resuspended in 100μl of TE. 50μl of water saturated phenol was added and the contents vortexed for 30 seconds. The contents were kept at room temperature for 15 minutes and the contents vortexed for 30seconds. The mixture was spun at 9000g for 5 minutes and the top aqueous phase removed. 10μl of 4M lithium chloride and 2.5 volumes of ethanol were added to the aqueous phase and the contents mixed. The mixture was kept at -70^0C for 20 minutes and the DNA pelleted by centifugation at 9000g for 10 minutes. The DNA pellet was resuspended in 20μl of 1mM Tris, 0.1mM EDTA pH 8.0. This DNA was subsequently used for sequencing. During the initiation of this work sequencing was conducted at Moredun Research Institute using the T7 Di-deoxy chain termination technique. Subsequent sequencing was kindly conducted by Mr Ian Bennet at the DNA sequencing facility, Department of Veterinary Pathology, Summerhall, University of Edinburgh.

2.5.8. Preparation of plasmid double stranded DNA for automated sequencing

This procedure and reagents were based on a kit provided by Promega in a "Wizard DNA purification kit" (Cat.Number A7100). Single colonies were picked from LB plates and grown overnight in 10ml of LB broth containing 50μg/ml ampicillin. The cells were pelleted by centrifugation at 10,000xg for 10 min. and the supernatant

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discarded. The cell pellet was resuspended completely in 400μl of cell suspension solution and transferred to a 1.5ml microfuge tube. 400μl of cell lysis solution was added and the contents mixed by inverting the tube 4 times. 400μl of neutralisation solution was added to the clear suspension and the contents mixed by inverting the tube 4 times. The lysate was spun in a microcentrifuge at 10,000xg for 10 min. and the supernatant transferred to a barrel of a minicolumn/syringe assembly containing 1ml of resuspended resin. The stopcocks were opened and vacuum applied to pull the resin/lysate mixture through the column. The vacuum was then released and 2 ml of column wash solution added to the barrel. Vacuum was applied to draw the solution through the minicolumn and the resin dried by continuing to draw vacuum for a further 30 seconds after the wash solution had been drawn through the column. The syringe barrel was removed and the minicolumn transferred to a microcentrifuge tube. The minicolumn was spun at 10,000xg in the microcentrifuge tube to remove excess wash solution and the minicolumn transfered to another microfuge tube. 50μl of water was added into the minicolumn and the tube centrifuged for 30 seconds to elute the DNA. The concentration was adjusted to 250ng/μl and the plasmid DNA sequenced using forward and reverse primers.

2. 5. 9 Di-deoxy chain termination sequencing of DNA using T7 polymerase

T7 polymerase sequencing was carried out using commercially available T7 sequencing kit (Pharmacia), which provided all the necessary reagents to carry out all the reactions by the dideoxy termination method of Sanger et al (1977), except for the [^{35}S] dATP, which was obtained from Amersham. All reactions were carried out

according to the manufacturers instructions. Plasmid DNA was sequenced to verify that the insert contained the desired viral DNA sequences.

Approximately 1μg per reaction was first denatured by adding NaOH to a final concentration of 0.4M NaOH and incubating for 10 min at room temperature. The DNA was then precipitated by the addition of 0.3 volumes sodium acetate, 0.7 volumes distilled water and 2 volumes ethanol. The DNA was pelleted and redissolved in 50μl of water. 2 μl of "annealing buffer" (a buffer solution containing $MgCl_2$ and DTT) and 1 μl of primer were added to the DNA, mixed gently, then incubated at 65^0C for 2 min then left to anneal while cooling to room temperature. "An enzyme premix" was prepared by adding 3 units of T7 polymerase in "enzyme dilution buffer" (Buffer solution containing Glycerol, BSA, and DTT) to 1 μl (10μCi) of [^{35}S] dATP, 3 μl of "labelling mix A" (dCTP, dGTP, dTTP in solution) and 1 μl of distilled water. 6 μl of "enzyme premix" was added to the annealed template and primer and incubated for 5 min at RT during which time the newly synthesised DNA was labelled by incorporation of [^{35}S] dATP. Chain termination was effected by the addition of 4 μl from this reaction to each of 4 tubes containing 2. 5 μl of "G", "A", "T", and "C" mixes respectively (each mix contained deoxy and di-deoxy forms of the respective base) and incubating for 5 min at 37^0C. 5 μl of "stop solution" was added to each tube prior to storage at -20^0C.

The sequencing samples were resolved by PAGE. 6% acrylamide gel solution was prepared from a 40% acrylamide stock (38% (w/v) acrylamide, 2% bis-acrylamide) with urea added to a final concentration of 7M and 10X TBE at a dilution of 1: 10. 1% (w/v) APS and 0.05% (w/v) TEMED were added immediately before pouring the gel. Electrophoresis in 1X TBE buffer was carried out using the S2 (BRL) apparatus. The gels were pre-run for 30 min before loading freshly denatured samples (heated to

80^0C for 2 min). The gels were fixed by immersing in 10% (v/v) methanol, 10% (v/v) ethanoic acid then transferred to Whatman 3MM filter and dried under vacuum at 80^0C for 30 min.

Detection of [^{35}S]- labelled nucleic acids in sequencing gels was achieved by exposure to medical X-ray film (Fuji) in radiography cassettes. The X-ray film was developed and fixed in "Photosol" CDL8 developer and "Photosol" CF40 fixer (Genetic Research Instrumentation Limited). Automated sequencing was performed at the Royal Dick Veterinary School, Summerhall on a LICOR machine.

2.5.10 Computer analysis of DNA sequences

The sequence analysis software package of the University of Wisconsin Genetics Group (UWGCG) was used. The package was available to the User Group, Daresbury Laboratory, Daresbury supported by Vax 3600 mainframe computer.

2. 5.10.1. Data Base Searching

The FastA programme (PEARSON) was used to match DNA sequences with the sequences contained in the EMBL and GENBANK databases.

2.5.10.2 Data sequence comparisons

The GAP and FastA program (UWGCG) were used to make comparisons between two sequences and to construct optimal alignment by inserting gaps to maximise the

number of matches. BESTFIT programme was used to determine homologies between cloned DNA fragments and AHV1 sequences in the databases.

2. 5. 11 DNA restriction enzyme analysis

DNASTAR set of programs was used to construct restriction maps of a viral DNA

2. 5. 12 Agarose gel electrophoresis of DNA

Nucleic acid grade agarose (BRL) was used at 1% concentration in TBE buffer. Electrophoresis was carried out at 1-4 Volts cm^{-1} until the required resolution was achieved and DNA fragments were visualised by fluorescence of the intercalated ethidium bromide in the U/V light of 302nm wavelength. DNA size estimation was achieved by comparison with the 1kbp ladder (GIBCO-BRL)

2. 5. 13 Quantification of nucleic acids

Nucleic acid was quantified by spectrophotometry. DNA and RNA have characteristic UV spectra, the peak of absobance is at 260nm, while the absorbance at 280nm is a measure of contaminating protein that is present (Sambrook et al 1989). The absorbance at 260 nm gives a measure of the concentration of nucleic acid in the sample. An absorbance of 1 ODU_{260} corresponds to a concentration of 50μg ml^{-1} for double stranded DNA and 40μg ml^{-1} for RNA. The ratio OD_{260}/OD_{280} is approximately 1.8 and 2.0 for clean DNA and RNA respectively.

2. 5. 14 Extraction and purification of DNA from gels

The method and reagents used were based on a "Geneclean^R Kit" (Stratatech Scientific). The piece of gel containing the DNA was weighed, and 4. 5 volumes of NaI and 0.5 volumes of TBE inhibitor added. The agar was melted at 55^0C for 5min. 10 μl of silica were added and the suspension placed on ice for 5 min. The suspension was then spun at 9000g for 30 sec and the pellet washed three times in 0.5 ml of Easiclene washing solution. The mixture was then pelleted by centrifugation at 9000g for 30sec. The pellet was finally resuspended in 30 μl of water and incubated at 55^0C for 5min. The DNA was recovered from the supernatant following centrifugation. This DNA could then be ligated to plasmid or labelled for use as a probe.

2. 5. 15 Polymerase chain reaction (PCR)

Two procedures were used to amplify sequences in DNA. Primers used with either protocol were purchased from Oswel or MWG-Biotech GmbH. Various oligonocleotide sequences were used to amplify different fragments of viral DNA. Primers used to amplify fragments to be used in ligation reactions were engineered so that they contained useful restriction sites. Pairs of primers for amplifying specific fragments of the AHV-1 genome are described when appropriate.

2.5.15.1 PCR using *Taq*

Amplification reactions were performed according to the methods described by Saiki et al (1985 and 1988). PCR was set up by adding 0.2 mM dNTPs, 0.2 mM of each pair of primers, 1x PCR buffer (10 mM Tris-HCl, 1. 5 mM $MgCl_2$, 50 mM KCl, 0.1mg ml^{-1} gelatine) and 2. 5 units of Taq polymerase enzyme (buffer and enzyme supplied by Boehringer Mannheim) to the DNA. The reaction volume was adjusted to 50 μl with water. Target DNA was 50 ng of AHV-1 genomic DNA or 2 μl of cDNA from the reverse transcriptase step (Chapter 3). The reactions were overlaid with 50 μl of mineral oil before being amplified using a Hybaid Omnigene thermal cycler.

2.5.15.2. Amplification of DNA using the Expand ™ Long Template PCR System

The Boehringer Mannheim Expand™ Long Template system reagents were used for PCR according to the manufacturer's conditions. This method was used to amplify long (over 1 kb) sequences. This method differs from the conventional PCR in that the system uses an enzyme mix containing the thermostable *Taq* and two *Pwo* DNA polymerases. PCR was set up by adding 0.35 mM dNTPs, 0.3 mM of each pair of primers (MWG- Biotech GmbH) specific for each viral DNA fragment to be amplified. 1x PCR buffer (10 mM Tris-HCl, 1.75 mM $MgCl_2$,) and 2.5 units of *Taq* polymerase enzyme the DNA. The reaction volume was adjusted to 50 μl with water.

Elongation steps were performed at $68^{0}C$ and the elongation times were varied according to the length of the required product.

The cycle used was similar when amplifying all templates, the only difference being the annealing temperatures. The cycle used is detailed in Table 2.5

TABLE 2.4. Cycle used in the Expand long template PCR

Temperature ^{0} C	Time	cycles
92	2 minutes	1
92	10 seconds	1
57	30 seconds	1
X	5 minutes	10
92	10 minutes	1
57	30 seconds	1
68	5 minutes	25
92	10 seconds	1
68	7 minutes	1

X= Annealing temperatures for different pairs of primers. These are given for each specific amplification reaction when appropriate.

CHAPTER 3

EXPRESSION OF PEPTIDE 1 AND PEPTIDE 5 *IN VITRO*

3.1 INTRODUCTION

Most of the studies on AHV1 virus have been conducted using an isolate (C500) derived from a cow showing clinical MCF (Plowright et al, 1975) and an isolate (WC11) obtained from a one week old wildebeest calf (Plowright et al, 1963). The WC11 isolate was shown to become cell free but still retained virulence for cattle following passage in cell culture. Further passage in culture resulted in attenuation of the virus. The attenuated virus however did not protect cattle following challenge with virulent virus. Attempts at developing vaccines using conventional methods have been unsuccessful (discussed in Chapter 1). Developments in molecular biological techniques have resulted in studies being carried out to elucidate the structure and genome organisation of the AHV-1 DNA (Bridgen et al, 1989, Handley et al, 1995).

The first characterisation of the AHV1 genome was conducted by Bridgen et al (1989). They showed that the genome comprises 130kbp of unique DNA flanked by up to 30kb of 1kb direct terminal repeats. Handley et al (1995) cloned and sequenced DNA from the unique region adjacent the terminal repeats of the attenuated and the virulent strains of AHV1. Differences in nucleotide sequences were observed between the clones derived from virulent and attenuated viral DNA. DNA and amino acid analysis of these clones revealed nine putative proteins. One polypeptide, designated protein 5 (P-5) was truncated in the attenuated virus. This ORF, of 496bp, was compared to all other sequence in the databases and no significant homology

was observed. The P-5 ORF was shown to contain a region coding for 100-130 amino acids that was highly antigenic. The ORF was positionally equivalent to the transforming gene of herpesvirus saimiri (HVS) subtype A strain 11(STP-A11) (Beisinger et al, 1990). Passage of HVS in culture results in the deletion of STP-A11 gene that is associated with transformation (Biesinger et al 1990, Murphy et al, 1989). It was therefore suggested that this protein could be responsible for virulence.

Another open reading frame, encoding a hypothetical polypeptide designated polypeptide 1, consisting of 300 amino acids, was common to the virulent isolate, low passage virulent derivative and the attenuated virus. This ORF was also shown to be present in OHV-2 induced cell lines (LCL) (Handley, 1993). Analysis of this ORF showed limited structural homology to the Tyrosine interacting kinase protein (TIP) of HVS, STP-A11 (Beisinger et al, 1992). The presence of two copies of this reading frame however indicated that this protein may not have a similar functional role (Handley, 1993). The presence of this ORF in the virulent and the attenuated viruses and also in immortalised cells suggests that it may be useful in diagnosis and expression studies.

A cell line with characteristics of large granular lymphocytes following cocultivation of lymph node and spleen suspensions from a rabbit infected with OHV-2 with feeder cell cultures was described by Reid et al, (1984). The cells were shown to be dependent on feeder monolayers and other substrates but gradually became independent of these substrates after prolonged culture. These immortalised cells, with characteristics of large granular lymphocytes derived from an OHV-2 infected rabbit are virulent for rabbits without any evidence of virus particles or antigens (Reid et al 1989, Reid et al, 1984). Similar cells are readily produced from tissues derived from C500 infected rabbits (Reid, personal communication). The ability to

establish LCL immortalised by either AHV-1 or OHV-2 provides an invaluable *in vitro* system to study the molecular basis for virulence, most importantly in OHV-2 because it has never been isolated in culture.

The aim of this chapter is to determine whether polypeptide-1 (P-1) and polypeptide-5 (P-5) are expressed *in vivo* and *in vitro*, and to determine whether either or both of these polypeptides play any role in the final outcome of disease in infected animals. The objectives were:

(1) To clone and express P-1 and P-5 in suitable expression systems

(2) To use the expressed proteins to immunise rabbits and then challenge these rabbits to determine the protective potential of these polypeptides in rabbits.

(3) To use the rabbit antisera to determine whether these polypeptides are expressed in AHV-1 immortalised LCL and monolayer cell cultures productively infected with the virulent (C500) and the attenuated (WC11) virus.

(4) To use the antisera to determine whether P-5 and P-1 are expressed in wildebeest and infected cattle.

3. 2. METHODS

3.2.1 Virological methods

Propagation of LCL and cultivation of C500 and WC11 in bovine turbinate cultures was conducted by Dr H. Reid and Miss I. Pow, MRI (section 2.3.1). Preparation of viral DNA is described in section 2.3.2.

3.2.2 PCR to Amplify P-1 and P-5 sequences in AHV-1 DNA

The primers used to amplify the P-5 were engineered to contain a Pst I and a BamH I site. Primers used to amplify a 400bp internal segment of the P-1 sequence were engineered so that they contained a BamH I and an EcoR I restriction site. The positions of the primers in the AHV-1 genome are shown in appendix 6.1.

P-1 primers

BamH I
318; 5'- TAC**GGATCC**CCTGACATTTCATCTCTTTTG- 3'

EcoR I
317; 5' -ATA**GAATTC**TGATGTGGCAGATGCATCTAT- 3'

P-5 primers

BamH I
S21; (76590) 5' -CCTGC**GGATCC**CATGCTGCCTTCCCCGTAAA -3' (76619)

Pst I
S20; (77045) 5' -CCT**CTGCAG**ATATCCTGGTCTCGTAAGGTG -3' (77074)

PCR was also conducted with S21 and a primer;

T64; 5' –ATATTCTGGGGAGTATAAAG –3' which is upstream of S20 (77023-77004). The PCR would amplify a fragment of 433bp. This PCR reaction was conducted on cDNA obtained from reverse transcription of LCL (section 3.2.15). To amplify peptide 5 sequences, DNA was denatured at 95^0C, and annealed at 50^0C, and extension was carried out at 72^0C for 3 min. 30 cycles were performed. To amplify peptide 1 sequences, the DNA was denatured at 95^0C and annealed at 65^0C. Extension was done at 72^0C for 3 min and a total of 25 cycles were performed.

3.2.3 Cloning and expression of peptide 1 and peptide 5

PCR was used to amplify the relevant sequences coding for these proteins using purified viral DNA obtained from cell cultures infected with virulent AHV-1 strain C500. The PCR products were enzyme restricted to create ligation sites for insertion into plasmids and the recombinant plasmids used to transform competent bacteria. P-5 was inserted into pTRX and P-1 into pGEX.. pTRX recombinants were used to transform G1724 *E.coli* cells and pGEX plasmids were used to transform JM109 *E.coli* cells. Sequencing was conducted to confirm orientation of ligation and positive clones used for expression studies. Polyacrylamide gels were then run on bacterial lysates to confirm presence of the protein of interest. *E.coli* cells transformed with plasmids without inserts were used as negative controls.

3.2.4. Protein expression in *E. coli*

3.2.4.1 Expression of pGEX recombinant P-1 in JM109 cells

A single colony of cells transformed by pGEX or recombinant pGEX was grown overnight in 1ml of LB at 37^{0}C. The bacteria were then grown in 10ml of LB media until the OD was between 0.4 and 0.6. The bacteria were then induced by the addition of IPTG to a final concentration of 1mM. The cells were grown for a further 4 hr and the cells used for protein extraction.

3.2.4.2 Expression of recombinant P-5 in pTRX in G1724 cells

A single colony of bacteria transformed by pTRX or the recombinant pTRX was grown overnight at 30^{0}C in RM media. The cells were then grown in 10ml of induction media at 30^0 C until the OD_{600} was between 0.4 and 0.6. The cells were then induced by growing the bacteria at 37^{0}C after addition of tryptophan to a final concentration of 1mM. The cells were then allowed to grow for 4hr and the cells used for protein extraction

3.2.5 Extraction of proteins from *E coli*

3.2.5.1 Extraction by lysis

Bacteria were pelleted by centrifugation at 800g for 10min and resuspended in 1/10 volume of 50 mM Tris-HCl pH 8.0, 2 mM EDTA . Freshly made lysozyme solution (10mg/ ml) was added to a final concentration of 100μg/ ml together with 0.1% (w/v) Tween 20 detergent. Lysis was allowed to occur at 30^{0}C for 30min . The lysate was then made 8mM with respect to $MgCl_2$ and the nucleic acids digested by addition of 5μl DNAse 1 (10U/ml) and 5μl of RNAse (10μg/ml) for 30 min at 0^{0}C. The lysate was centrifuged at 10,000g for 5min. The supernatant was harvested and the pellet resuspended in 100μl of water. Both the pellet and the supernatant were analysed for the presence of the protein of interest.

3.2.5.2 Extraction by sonication

Bacteria were pelleted by centrifugation at 800g for 10min and the cells resuspended in 1/50 of the original volume of either 1 mM Tris, or 1x MOPS, or 1 mM HEPES, all of these buffers containing 1 mM β-Mercaptoethanol, 1 mM p-APMSF. The cells were then sonicated by 3 cycles of 10 seconds using a Heat systems Ultrasonic, Inc. Model W-385 set at maximum output. The sonicate was centrifuged at 10,000g for 5 minutes. The supernatant was kept and the pellet resuspended in the original volume of water. Both the supernatant and the pellet were assayed for protein by electrophoresis.

3.2.6 Polyacrylamide gel electrophoresis of proteins

Electrophoresis was based on the method of Laemmli, (1970) using "Mini" or "Maxi" Protean apparatus (Biorad). Either 15% or 10% acrylamide gels were prepared using the appropriate volumes of 30% acrylamide:10% SDS: 1.5M Tris-HCl, pH 8.8 (resolving gel) or 1M Tris-HCl pH 6.8 (stacking gel): 10% freshly made ammonium persulphate: TEMED: H_2O. Samples were diluted 1:1 with loading buffer (50 mM Tris-HCl pH6. 8, 25 mM SDS, 0.1 M Bromophenol blue, 10% glycerol, 100 mM DTT) and boiled for 5min before loading into wells. Gels were then run at a constant voltage setting of 100V for 2-3hr. The gel was removed and the proteins in the gel transferred to membranes or stained. Proteins were visualised by staining with Coomassie blue. Molecular weights were determined by running samples in parallel with protein molecular weight markers (Sigma) of known concentration.

3.2.7 Western blotting

3.2.7.1 Transfer of protein onto membranes

Gels were removed from Protean tanks and equilibrated in transfer buffer (10mM Tris, 100mM Glycine, 25% (v/v) methanol) for 15 min. PVDF western blotting membranes (Boehringer Mannheim) were placed in methanol for 30sec and rinsed in water for 5min. The membranes were then equilibrated in transfer buffer (250ml Methanol in 750ml 1mM Tris-HCl, pH 8.0, 200mM Glycine) for 5min. The method used was the semi-dry method using apparatus supplied by BIORAD. Whatman 3MM filter previously wetted in transfer buffer was placed on the anode electrode. The PDVF membrane was then placed on top of the paper and gel placed on the membrane. Finally, a buffer- wetted 3MM was placed on the gel. The apparatus was then assembled and transfer performed at 150mA for 20-30min. The filter was stained in Ponceau and rapidly destained with water to visualize the proteins. The membrane could then be used directly for blotting or air-dried and kept at 4^0C until required.

3.2.7.2 Antibody detection of protein

PVDV filters were placed in blocking solution (washing buffer containing 10% milk powder) for 1hr at room temperature with gentle agitation and then washed three times for 15min each in washing buffer (1x TBS, 0.01%Tween). The filter was then incubated in primary antibody diluted 1: 40 for P-5 and P-1 antisera, and

1:5000 for mouse anti-thioredoxin monoclonal antibody (Invitrogen) or anti-GST monoclonal antibody in blocking solution for 1hr at room temperature with gentle agitation. The membrane was washed again for 15 min with three changes of washing solution. The membrane was then incubated for 1hr at room temperature with secondary antibody [sheep anti-mouse IgG, (Sigma) or donkey anti-bovine IgG (SAPU) conjugated to horse radish peroxide] diluted (1: 2000) in blocking solution. The filter was washed again for 15min with three changes of washing solution and then rinsed in water for 5min. 20 ml of TMB substrate (Sigma) were added per $100cm^{-2}$ of filter and the membrane placed in the dark for 10min for colour to develop. The reaction was stopped by washing the membrane with water for 5min. The filter was used for photography or air-dried before storage at room temperature.

3.2.8 Electroelution of proteins from gels

Proteins were run on a preparative "mini" or "maxi" Protean gel apparatus (BIORAD) in parallel with markers. A longitudinal strip of gel containing the desired protein was excised with a scalpel blade and placed in a Petri dish containing 5 ml of cathode buffer (0.01M Tris-HCl, pH 8.0, 0.05% SDS). The gel was cut into small (approximately 2 mm) blocks and these pieces were placed in the cathode chamber of the electroeluter apparatus (Genetic Research Instruments). Anode buffer (0.01M Tris-HCl, pH 8.0 , 0 05M Glycine) was placed in the anode chamber until the buffer flowed into the cathode tank. Both tanks were then carefully filled with anode buffer without disturbing the gel pieces and buffer in the cathode tank. Electroelution was then performed at 50V for 12 to 16 hr. The bottom 5 ml of the anode buffer was then retained and placed in a Centricon[R] tube with a molecular wt. cut off of 30,000 kDa

(Amicon). The Centricon tube was then centrifuged at 3000g for 1hour to concentrate the protein.

3.2.9 Immunisation of rabbits

Each adult New Zealand white rabbit conventionally reared at MRI was immunised by intramuscular (i/m) inoculation of 1 ml of a 1:1 (v/v) emulsion containing 50μg of protein and Freund's complete adjuvant. Each rabbit was boosted i/m again with a 1 ml of a 1:1 (v/v) emulsion containing 50μg of protein and Freund's incomplete adjuvant after 28 days. The rabbits were then inoculated intravenously 14 days later with 50μg of protein without adjuvant (table 3.1). Rabbits were bled for serum before each inoculation and the serum used for antibody assay. A large volume (approximately 100ml) of blood was collected 10 days after the last inoculation.

<u>**TABLE 3.1: RABBIT IMMUNISATION AND CHALLENGE (C500)**</u>

Rabbit No.	Protein	Date immunised	Date challenged	Remark
21&22	P-5	11/7/96, 8/8/96, 8/10/96	17/1/97	Killed 31/1/97
27	P-1	8/10/96, 4/11/96 19/11/96	17/1/97	Killed 30/1/97
28	P-1	8/10/96, 4/11/96 19/11/96	17/1/97	Killed 22/1/97

3.2.11 Indirect immunofluorescence antibody test

This test was performed to detect P-5 and P-1 antigens in monolayer cell cultures grown on cover slips infected with virulent (C500) and attenuated (WC11) strains of AHV-1 and also in LCL immortalised by OHV-2 and AHV-1. Bovine turbinate cells grown on cover slips were infected at a multiplicity of infection of 0.1 and the cells fixed when the cytopathic effect was about 50% by placing the coverslips in cold acetone at 4^0C for 10 minutes. The coverslips were mounted onto glass slides, cells uppermost, with DPX. Such coverslips could be used immediately or stored at -20^0C until required. Cytospin preparation of LCL grown in suspension or peripheral blood lymphocytes obtained from cattle experimentally infected with either AHV-1 or BHV-1 were adjusted to 2-5 x 10^6/ ml in Minimum Essential Media (MEM) (supplied by GIBCO). Two hundred microlitres of these cells were spun for 5 minutes at 1500rpm in a Cytospin (Shandon, Cytospin3) and the cells on the glass slides fixed in acetone for 10 minutes and then treated as the coverslip cells mounted on slides.

To reduce non-specific binding, cells were blocked by flooding with a 1:20 dilution of horse serum and incubating at 37^0C in a moist chamber for 30 minutes. The cells were then washed for 10 minutes with two changes of PBS, air dried and 50µl of various dilutions of rabbit serum added onto the cells. The coverslips were incubated again at 37^0C for 30 minutes and the cells washed as before. The cells were stained by adding 50µl of 1:40 dilution of goat anti- rabbit FITC conjugate (Sigma) onto the coverslips and the slides incubated as before. The cells were washed for 10 minutes with two changes of PBS and the cells air dried. The coverlips were then examined under UV microscope at 1:40 magnification.

3.2.11 ELISA Test

This test was performed to detect and assay P-5 and P-1 antibodies in immunised rabbits, experimentally infected cattle and wildebeest sera. ELISA (Dynatech) plates were coated with 50μl of protein (1μg) in coating buffer and the plates incubated overnight at 4^0C. The contents were discarded and the wells washed 5 times by flooding with coating buffer. 50μl of antibody dilutions in coating buffer containing 10% (w/v) milk powder was added into each well and the plates incubated for 30 minutes at room temperature. The contents were discarded and the plates washed again with 5 changes of washing buffer. 50μl of anti-rabbit immunoglobulin Horse Radish Peroxide conjugate (SAPU) diluted 1: 1000 in washing buffer containing 10% (w/v) milk powder. Donkey Anti-bovine IgG (Sigma) was used for both cattle and wildebeest sera and sheep anti-rabbit IgG with rabbit sera. The conjugate was removed and the plates washed again with 5 changes of washing buffer and 50μl of conjugate (25.2 mg ortho-phenyldiamine tablet and 268mg phosphate-citrate buffer with urea hydrogen peroxide tablet, Sigma) added into each well. The plates were then incubated in the dark at room temperature for colour to develop. The reaction was stopped by adding 50μl of 2.5M H_2SO_4 and antibody levels determined by colorimetry using an ELISA reader at a wavelength of 492nm.

3.2.13 Extraction of RNA from LCL or Peripheral blood lymphocytes (PBL)

This was a modification of the method described by Chomczynski and Saachi (1987). Extractions were done using the protocol and reagents from an "RNA Extraction Kit" (Stratagene). PBL were recovered from blood in EDTA obtained

from cattle experimentally infected with either AHV-1 or BHV-1(section 3.2.22). EDTA blood was spun at 1800x g for 10 min and the buffy coat layer removed. The buffy coats were resuspended in 10ml of PBS and the cells pelleted by spinning at 1800x g for 10 minutes. The cells were washed again in PBS and 10^8 cells resuspended in 10ml of freshly made denaturing solution, guanidine isothiocyanate (GITC) supplied in the kit. LCL grown in suspension were pelleted by centrifugation at 800g for 10 minutes and washed once with PBS. 1x 10^8 cells were then resuspended in 10 ml of freshly made denaturing solution. The PBL or LCL were left at room temperature for 1min. One ml of 2M sodium acetate (pH 4.0) was then added and the contents mixed by inversion. Ten mls of water saturated phenol was added to the mixture and the contents vigorously mixed by inversion. Finally 2.0 ml of chloroform: isoamyl alcohol (24:1) was added and the contents vigorously mixed for 10 sec. The mixture was placed on ice for 15min and then centrifuged at 10000g for 20min at 4^0 C. The aqueous phase was removed and mixed with an equal volume of isopropanol. The mixture was cooled at -20^0C for 1hr and the RNA precipitated by centrifugation at 10000g for 20min at 4^0C. The RNA pellet was resuspended in 3 ml of denaturing solution and an equal volume of isopropanol added. The mixture was incubated at -20^0C for 1hr and then the RNA was pelleted by centrifugation at 10000g for 10min at 4^0C. The RNA was resuspended in sterile water and the concentration adjusted to 100 ng/ml or 500 ng/ml after quantification by UV spectrophotometry.

3.2.14 Agarose gel electrophoresis of RNA

Agarose gels were prepared by dissolving 1g of "ultra pure" agarose in 1x MOPS by boiling. The mixture was allowed to cool to 50^0C and then formaldehyde was added to a final concentration of 7.5%. RNA samples were diluted 1:4 in RNA loading buffer and then mixed briefly by vortexing. The RNA was denatured at 65^0C for 5 min and chilled immediately on ice before loading into the wells. The gel was run at 5V cm^{-1} for 1hr and the RNA visualised using a UV transilluminator.

3.2.14 cDNA synthesis

cDNA synthesis was conducted using reagents contained in a commercial kit (Invitrogen). The method used was based on instructions of the manufacturer. Ten μg of RNA (extracted from LCL or PBL) in 11.5 μl of water were placed in a microfuge tube and 1 μl of random hexamers added. For a control reaction, 1μg of "kit" MS2 mRNA and a specific 17mer primer was used. The sample was heated to 65^0C for 10min and then allowed to cool at room temperature for 2min. The following reagents were then added in the order indicated; 1 μl RNAse inhibitor, 4 μl 5x RT buffer (250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM DTT pH 8.5), 2 μl 100 mM dNTPs, 0.5 μl reverse transcriptase enzyme. The contents were mixed gently and incubated at 42^0C for 1hr. The sample was then used for PCR.

3.2. 15 Southern transfer of DNA onto membranes

This method was used to transfer DNA from agarose gels onto Hybond N (Amersham) membranes. The gel was placed in denaturing solution (0.5M NaOH, 1.5M NaCl) and incubated for 30 min. The gel was then briefly rinsed in distilled

water and placed in transfer buffer (1M NH_4Cl, 0.02M NaOH) for 1hr. Nylon membrane previously soaked in the transfer buffer was then placed on top of the gel and three sheets of Whatman 3MM, paper previously soaked in transfer buffer placed on top of the membrane. Finally a stack of paper towels were placed together with a 500g weight on top of the paper. Transfer was allowed to proceed overnight. DNA was then fixed to the membrane by cross-linking using a UV transilluminator for 1 min.

3.2.16 Preparation of digoxigenin labelled probes

Probes were made from P-5 and P-1 PCR products. P-5 probe was also made from restriction enzyme products of recombinant plasmid DNA. The procedure was performed according to the manufacturer's instructions with a slight modification. 300ng of template DNA was denatured by boiling for 10 min in a total volume of 15 µl and then chilled quickly on ice. 2 µl hexanucleotide mix were added followed by 2 µl dNTPs and 1 µl of Klenow enzyme provided in the "DIG DNA labelling kit". The reaction proceeded for 2hr at 37^0C and then stopped by adding 2 µl 0.2M EDTA.

3.2.17 Hybridisation of "DIG" labelled probes

Hybridisations were carried out as recommended by the manufacturer's (Boehringer Mannheim). Membranes were prehydridised by incubating in hybridisation buffer (5x SSC, 0.5% N-Laurylsarcosine, 0.2% (w/v) SDS, 1x blocking reagent, pH 7.0) (20 ml 100 cm^{-2} of membrane) for 1hr at 42^0C when using P-5 probe and 65^0 C when using P-1 probe. The solution was then decanted and fresh hybridisation buffer added

containing the probe which had been denatured by boiling for 5 min. Hybridisation was carried out for 2-16 hr at the appropriate temperature and the filters washed twice in 0.2xSSC, 0.1%SDS at the same temperatures for 30min.

3.2.18 Detection of bound "DIG" labelled probes

The detection of bound probe was performed using reagents and according to the manufacturer's (Boehringer Mannheim) instructions. Filters were incubated in blocking buffer (100 mM Tris-HCl pH 7.0, 150 mM NaCl) containing 0.5% blocking reagent, for 30min. The filters were then incubated for 1hr in blocking buffer (5 ml cm^{-2}) containing a 1:5000 dilution of alkaline phosphatase conjugate. The membrane was then washed twice in TN buffer (100 mM Tris-HCl, pH 7.0, 150 mM NaCl) for 15 min. The filters were placed in TNM (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 50 mM MgCl, pH 9. 5) for 5 min. 10 ml of TNM to which had been added 45 μl Nitro blue tetrazolium (NBT) and 35 μl X-phosphate were then added. The filters were kept static in the dark for 30min-4hr until the colour developed to the required intensity. The reaction was stopped by rinsing the filters in water for 5min after which the filters were air-dried.

3.2.19 Virus isolates used to infect cattle

The virulent C500 isolate (Plowright et al, 1975) used was kindly provided as a cell culture infected flask by Dr. H. Reid. The cells were harvested by trypsinisation when cpe covered approximately 50% of the cell sheet and resuspended in 10mls of cell culture media.

The WC11 strain (Plowright et al, 1965) used was an attenuated virus that had been previously passaged 49 times in bovine calf kidney cells and 50 times in bovine thyroid cultures. This virus was revived from -70^{0}C and passaged 3 times in bovine kidney cells.

The Bovine herpesvirus1 (BHV1) virus used was an Oxford strain (Dawson et al, 1962) that was revived from -70^0 C and passaged twice in bovine kidney cell cultures.

3.2.20 Titration of viruses

Cell free WC11 AHV-1 isolate and the Oxford strain of BHV1 were titrated before inoculation into cattle. Titrations were carried out in microplates. Twenty five microlitres of HEPES buffered growth media [(14mM HEPES in GMEM (GIBCO)] was dispensed into each well. Twenty five microlitres of a suitable dilution of virus was added into four wells. Additional doubling dilutions were then carried out. To each well was added 100µl of cell suspension containing 2x 10^5 cells/ml. The plates were then sealed, incubated at 37^{0}C and observed microscopically for cpe for up to 10 days after inoculation. Titres were calculated by the method of Reed and Muench (1938) and expressed as 50% tissue culture infective doses ($TCID_{50}$)

3.2.21 Virus neutralisation test

Virus neutralisation tests were carried out to screen sera from 1-2 year old cattle for AHV1 and BHV1 antibodies. These cattle were either Holstein Friesian or Zebu breeds. The tests were carried out in tissue culture microtitre plates using secondary

bovine kidney cells. Sera for testing was heat inactivated at 56^0 C for 30 minutes and diluted 1in 2 in MEM. 50μl of each test sera was added to 4 wells containing 100-200 $TCID_{50}$ of either virus (BHV-1 or AHV-1). Finally 100 μl of $2x10^5$ cells /ml was added to all wells. Controls were sera obtained from rabbits previously immunised with either virus that had been freeze-dried and stored at -20^0C. The plates were sealed and incubated at 37^0 C and read microscopically after 9 days. Only those wells showing cpe at a serum dilution of 1:4 were considered to be negative for antibody.

3.2.22 Inoculation of cattle

Two steers of the Zebu breed, seronegative for BHV-1 and AHV-1 were used for inoculation intravenously with 2ml of each virus strain or isolate. Virus titres were calculated using the method of Spearman-Karber (Lenette et al, 1969). The titre of BHV1 inoculum was 2.3 $x10^6$ $TCID_{50}$/ml and the titre of attenuated WC11 was 3.2 x 10^4 $TCID_{50}$/ml. The C500 virus isolate used was a suspension of infected bovine turbinate cells kindly provided by Dr. H. Reid.

3.3 RESULTS

3.3.1. Construction of P-5 clones

The 496bp ORF corresponding to polypeptide -5 described by Handley et al (1995) from C500 DNA was cloned in frame into the thioredoxin expression vector pTRX. The cloning sites were Pst I and BamH I and these were introduced into the viral DNA PCR primers.

The recombinant plasmids were used to transform G1724 *E coli* and the recombinant clones determined by their *Amp*$^{+}$ phenotype and confirmed by agarose-electrophoresis of the recombinants after digestion with Pst I and BamH I. Two recombinant clones, designated clones 11 and 12 were found to possess the P-5 insert and these were used in subsequent expression studies. Sequencing of the insert was performed to verify the presence of P-5 sequences in the insert.

3.3.2 Construction of P-1 clones

A 400bp fragment of the putative P-1 ORF that had been shown to be highly antigenic using the Fastscan program (Handley et al, 1995), was successfully cloned in frame into the BamH I and EcoRI sites of pGEX. The recombinant molecules were used to transform JM109 *E coli* grown in LB broth, and the recombinant clones determined by their *Amp*$^{+}$, *LacZ*$^{-}$ phenotype. Small scale DNA preparations were made from these clones and the insert sizes determined. Sequencing of the insert was performed to confirm presence of the P-1 sequences.

3.3.3 Expression of P-5

To determine whether the recombinant clones were able to express the foreign C500 virus sequences, cellular protein was extracted from induced G1724 cells by lysis or sonication and analysed in acrylamide gels. Results showed that the fusion protein was only present in the insoluble fraction of the lysates or sonicates whereas the control thioredoxin protein was present in the supernatant (Figure 3.1). Both methods of extraction however gave similar and reproducible results. The recombinant protein

was 29K whereas the thioredoxin was 12K. Attempts to increase the solubility of the fusion protein by inducing expression at low temperatures (18^0C and 30^0C) were unsuccessful. A strain of *E coli,* G1624 which is almost isogenic to G1724 but expresses fusion protein at less than 30^0C was also used but the P-5 fusion protein was found to be insoluble.

To confirm that protein band at 29K in the induced cells was indeed the fusion protein, western blots were made from induced and uninduced lysates from transformed cells. The blots were reacted with a monoclonal antibody specific for the thioredoxin fusion partner (Invitrogen) (data not shown). Results indicated that the monoclonal reacted strongly with the recombinant protein and the thioredoxin protein but not with any bacterial proteins from the induced and uninduced *E coli*.

ELISA results using antisera from immunised rabbits indicated that the P-5 fusion protein was highly antigenic as the polyclonal sera reacted more strongly with the fusion protein as compared to the reaction between the thioredoxin and the polyclonal sera when both proteins were used at similar concentrations, ELISA (tables 3.2 and 3.3).

3. 3.4 Expression of GST-P-1 fusion

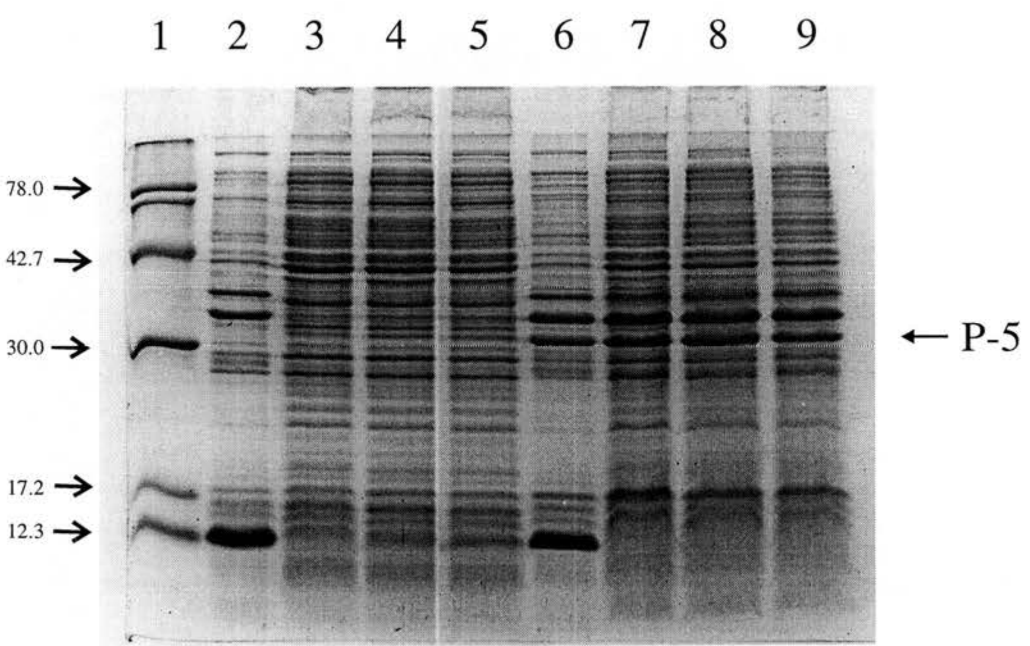
To determine whether the pGST-P-1 recombinants and pGST were expressed in IPTG-induced JM109 bacterial cells, the cells were lysed and the resulting pellets and supernatants analysed for protein on polyacrylamide gels. Protein profiles showed that both the GST and the P-1 fusion were soluble with molecular weights of approximately 27K and 42K respectively (Figure 3.2). Recombinant clones produced a protein of the same molecular weight as the GST but at a lower yield

suggesting GST or a protein of similar molecular weight is encoded chromosomally and is therefore produced endogenously by *E. coli*.

The recombinant protein appeared to be susceptible to degradation, presumably by proteases as this was inhibited by A-PMSF. Attempts to perform western blots using an anti-GST antibody (Pharmacia) have been unsuccessful as the anti-GST monoclonal antibody reacted with all bacterial proteins in the lysates.

The fusion protein was used to raise antiserum in rabbits. However, the sera obtained from the immunised rabbits could not be tested for anti P-1 antibody as the fusion protein was degraded very easily and could not be therefore used to coat plates in an indirect ELISA.

Figure 3.1 PAGE Showing the molecular wt.of P-5



Lane 1 Molecular wt. markers (kiloDaltons)

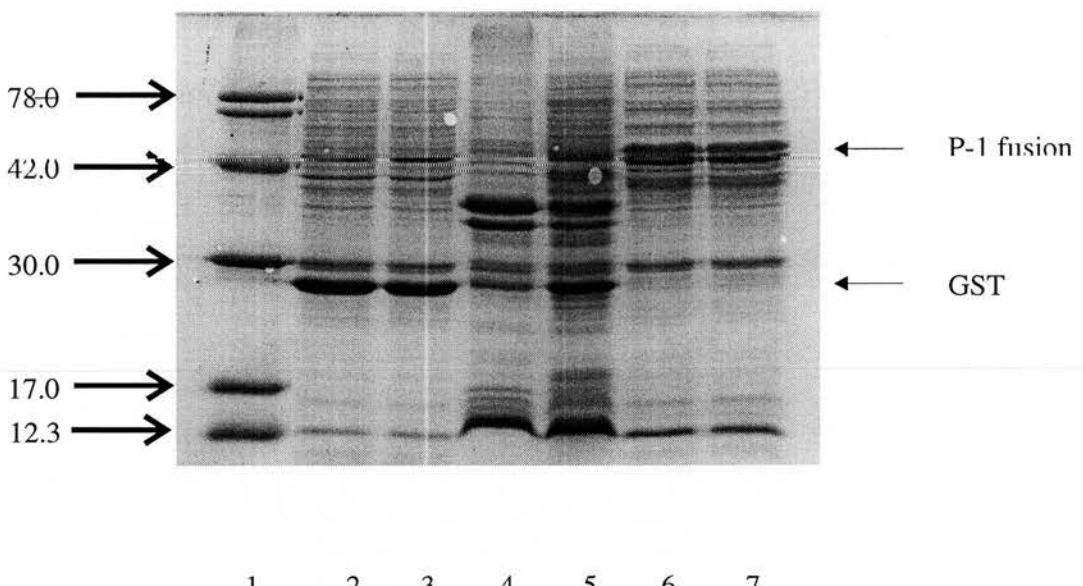
Lane 2 Uninduced cellstransformed with recombinant pTRX

Lane 3, 4 & 5 sonicates of of uninduced cells transformed with recombinant pTRX

Lane 6 Lysate of induced cells transformed with recombinant pTRX showing the P-5 fusion proteins

Lane 7, 8 &9 Sonicates of induced cells transformed with recombinant pTRX

Figure 3.2; PAGE Showing the molecular wt. of P-1 AND GST



Lane 1 - Molecular wt. markers(kiloDaltons)

Lane 2 & 3 - Lysates of induced pGEX transformed cells

Lane 4 - Lysates of uninduced pGEX transformed cells

Lane 5 - Lysates of uninduced P-1 and pGEX recombinant transformed cells

Lane 6 & 7 - Lysates of induced P-1 and pGEX recombinant transformed cells

3.3 5 Expression of P-1 and P-5 in LCL and PBL's

Both proteins were detected by indirect immunofluorescence in bovine turbinate cell infected with either C500 or WC11 as well as in LCL infected with either AHV1 and OHV2. Results for C500 and LCL are shown (Figure 3.3-3.10).The results showed that there was diffuse cytoplasmic fluorescence and particulate intranuclear fluorescence in BT cultures infected with the virulent C500 isolate when using the P-5 and P-1 antisera respectively. BT cultures infected with the attenuated WC11 isolate showed both intranuclear and cytoplasmic fluorescence and nuclear fluorescence when using P-1 and P-5 respectively. Uninfected cells did not show fluorescence.

Diffuse cytoplasmic fluorescence was seen in cells immortalised by either AHV1 or OHV2 when reacted with antisera to both P-1 and P-5.

No evidence of P-1 or P-5 was seen in peripheral blood lymphocytes obtained from cattle infected with either AHV-1 or BHV1.

3.3.6 PCR and RT-PCR on peripheral blood lymphocytes

Samples were collected before inoculation and then every 5 days until the C500 inoculated cattle showed pyrexia (described in section 3.3.8). Samples were collected on the day of fever and then daily for a further 3 days before the reacting animals were sacrificed. All blood samples collected after onset of pyrexia were AHV-1 PCR positive.

There was no evidence of P-5 or P-1 mRNA in lymphocytes obtained from cattle infected with either the attenuated or virulent virus. One sample obtained from a steer

inoculated with C500 was faintly positive. This was from buffy coat collected two days after onset of fever.

3.3 7 Transcription of P-1 and P-5 in LCL of OHV-2 and AHV-1

Synthesis of cDNA was successfully achieved from LCL immortalised by either OHV-2 or AHV-1 using the "Invitrogen kit". 2μl of this reaction mixture was used to amplify P-1 and P-5 sequences in the cDNA using specific primers for each ORF and the amplified products visualised in agarose gels. To confirm that the PCR was amplifying the cDNA and not contaminating viral DNA, a control experiment was also performed using RNA that was digested with DNAse and then phenol extracted before the RT step and the results showed that the DNA amplified was from the cDNA. PCR reactions were set up using the equivalent volume of LCL RNA. The results showed that there was trace amplification of viral DNA. However, the amount of DNA so amplified was insignificant when compared to the amount of DNA that was amplified using the cDNA.

Southern blots on the P-1 and P-5 PCR products were performed to confirm that the RT-PCR products were P-1 and P-5 (Figures 3.11 & 3.12). The results show that LCL transcribe mRNA for both proteins.

3.3.8 Reaction to infection and sampling of cattle used for P-5 ELISA

Cattle inoculated with the Oxford strain of BHV1 developed fever 8 days post inoculation (d.p.i.) whereas those animals inoculated with the C500 strain of AHV1 reacted 14d.p.i. Animals inoculated with the attenuated WC11 did not show any clinical disease.

The disease in BHV-1 infected cattle was characterised by a mild fever (39.1^0 C - 39.7^0 C), and ocular discharge. The animals recovered 4 days after onset of fever. The animals inoculated with C500 developed severe disease characterised by high fever 14 days post inoculation (40.5^0 C and 41.2^0 C). There was conjunctivitis which was accompanied by severe serous nasal discharge. The animals were killed 3 days after clinical disease and peripheral lymph node samples collected for virus isolation and PCR.

Serum samples were collected before inoculation and then on day 5 and 10. Sera was thereafter collected on day 14 when the C500 inoculated cattle reacted and on each of three subsequent days.

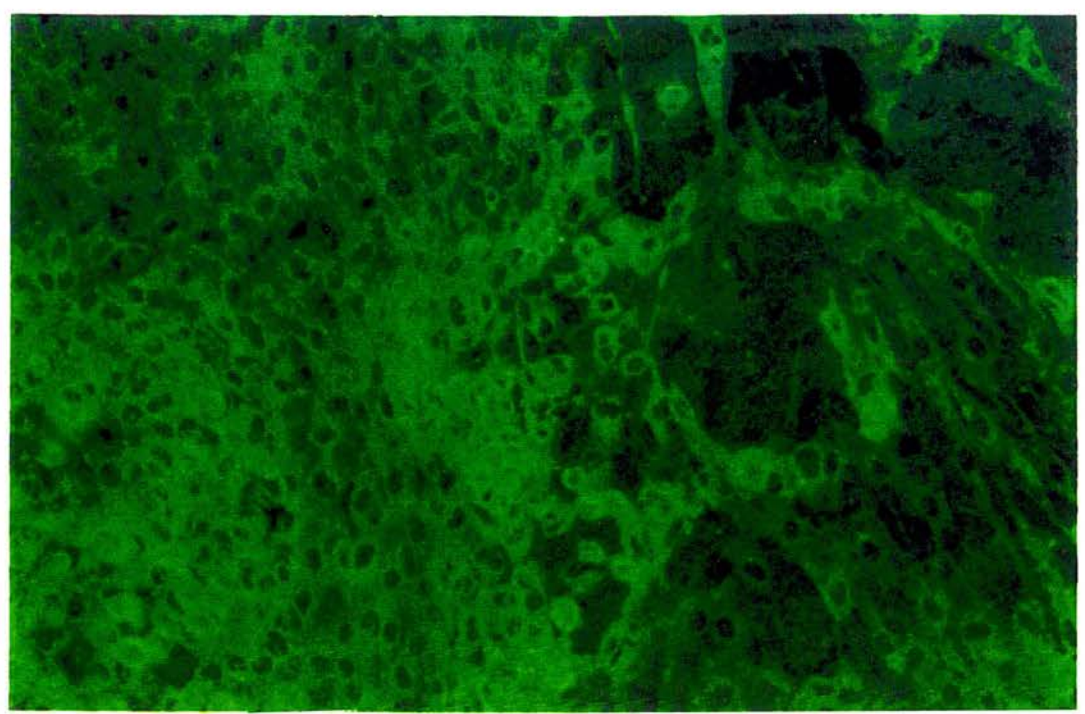


Figure 3.3. Diffuse cytoplasmic fluorescence in bovine turbinate cells infected with AHV-1 strain C500 in an indirect immunofluorescence (IIF) test using P-5 antiserum (x 100).

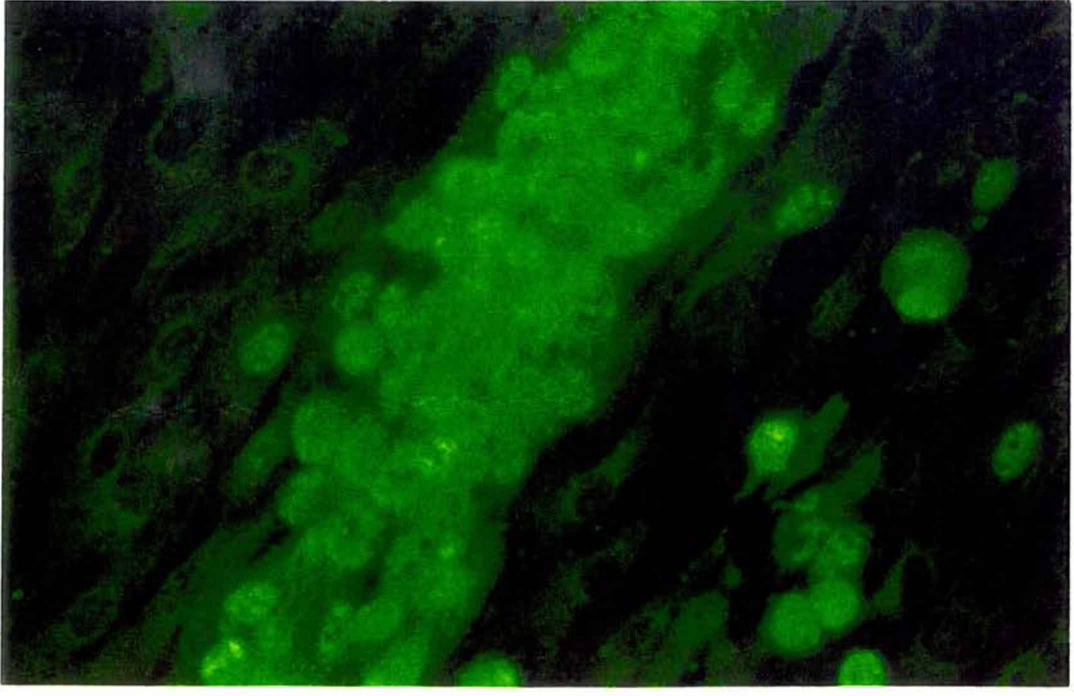


Figure 3.4. Particulate intranuclear fluorescence in bovine turbinate cells infected with AHV-1 strain C500 in an IIF test using P-1 antiserum (x 200).

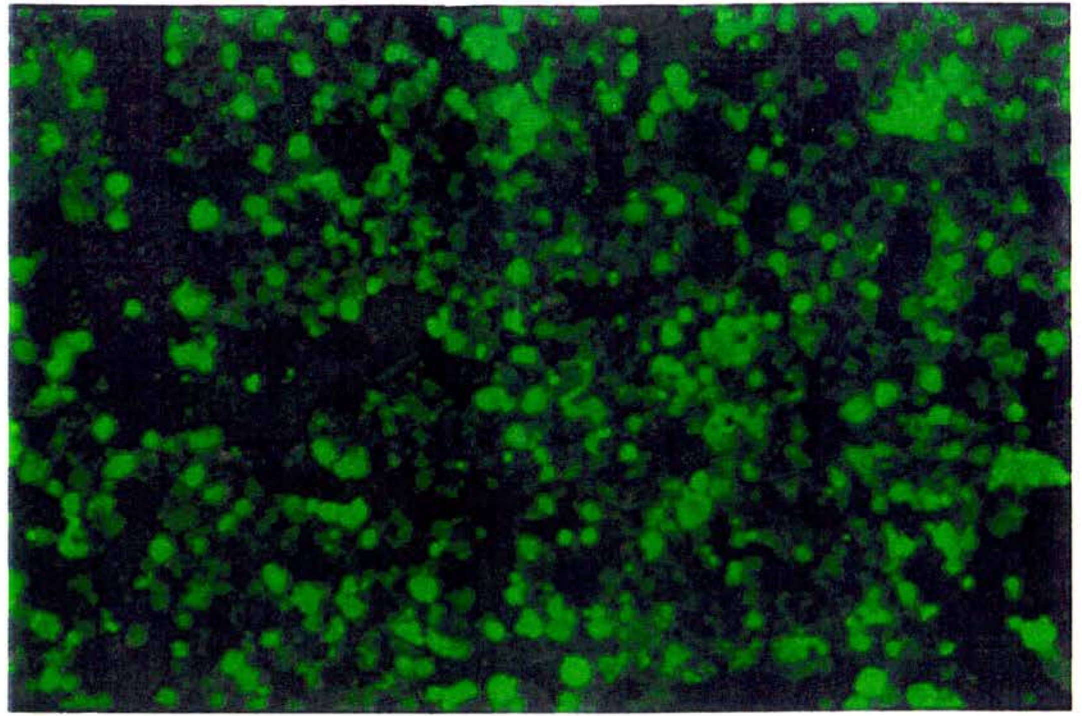


Figure 3.5. Diffuse cytoplasmic and nuclear fluorescence in AHV-1 LCL in an IIF test using a P-5 antiserum (x 200).

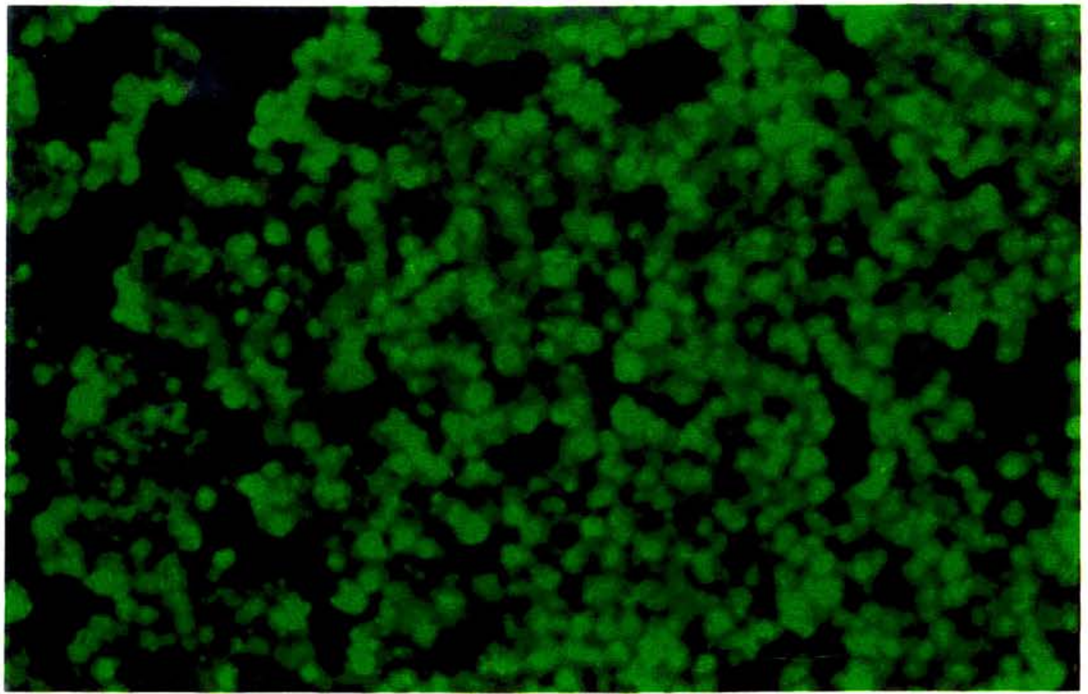


Figure 3.6. Diffuse cytoplasmic and nuclear fluorescence in OHV-2 LCL in an IIF test using a P-5 antiserum (x 100).

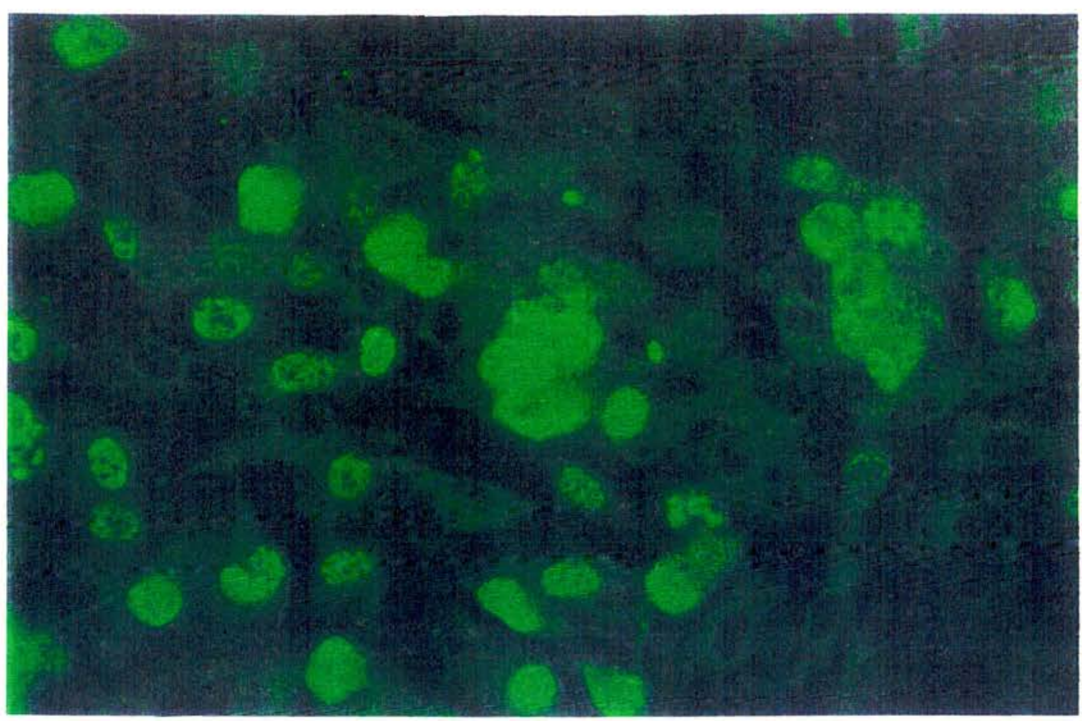


Figure 3.7. Particulate and diffuse intranuclear nuclear fluorescence in bovine turbinate cells infected with WC11 in an IIF using a P-1 antiserum (x 200).

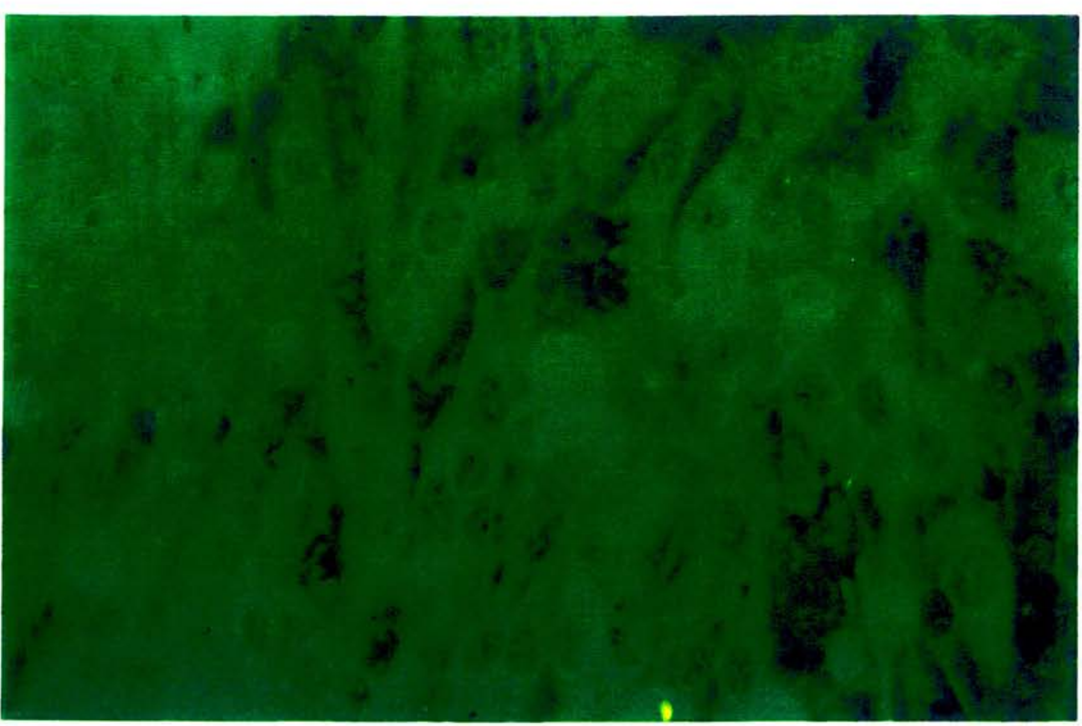


Figure 3.8. Diffuse cytoplasmic fluorescence in bovine turbinate cells infected with WC11 in an IIF using a P-5 antiserum (x 200).

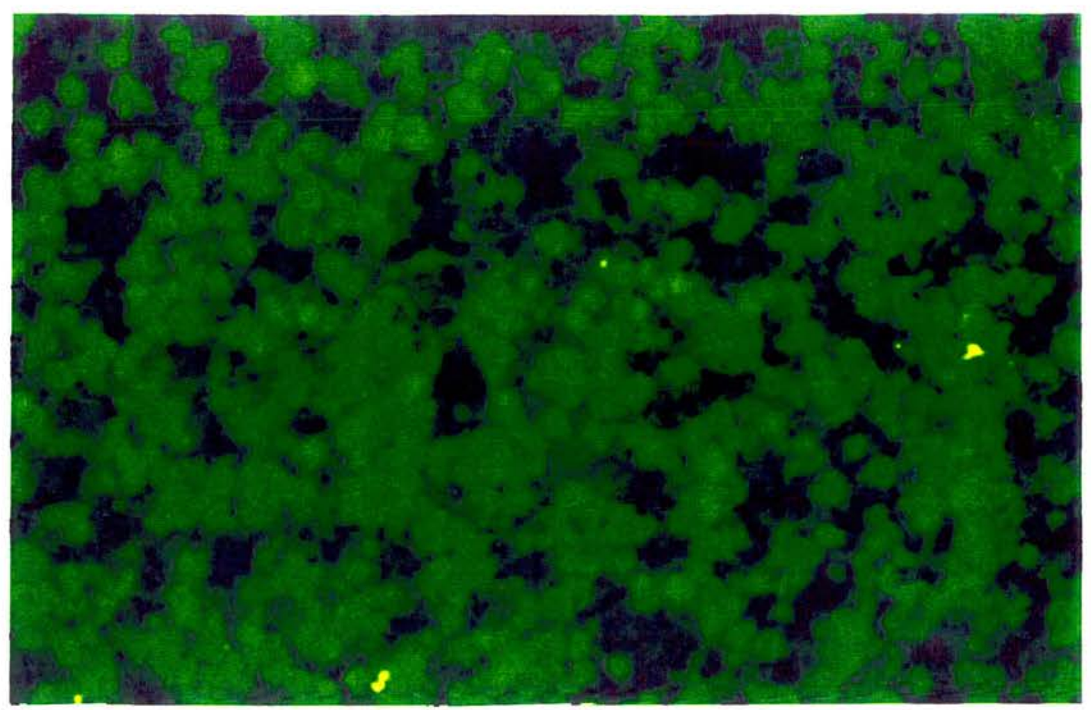


Figure 3.9. Diffuse cytoplasmic and nuclear fluorescence in AHV-1 LCL in an IIF using a P-1 antiserum (x 200).

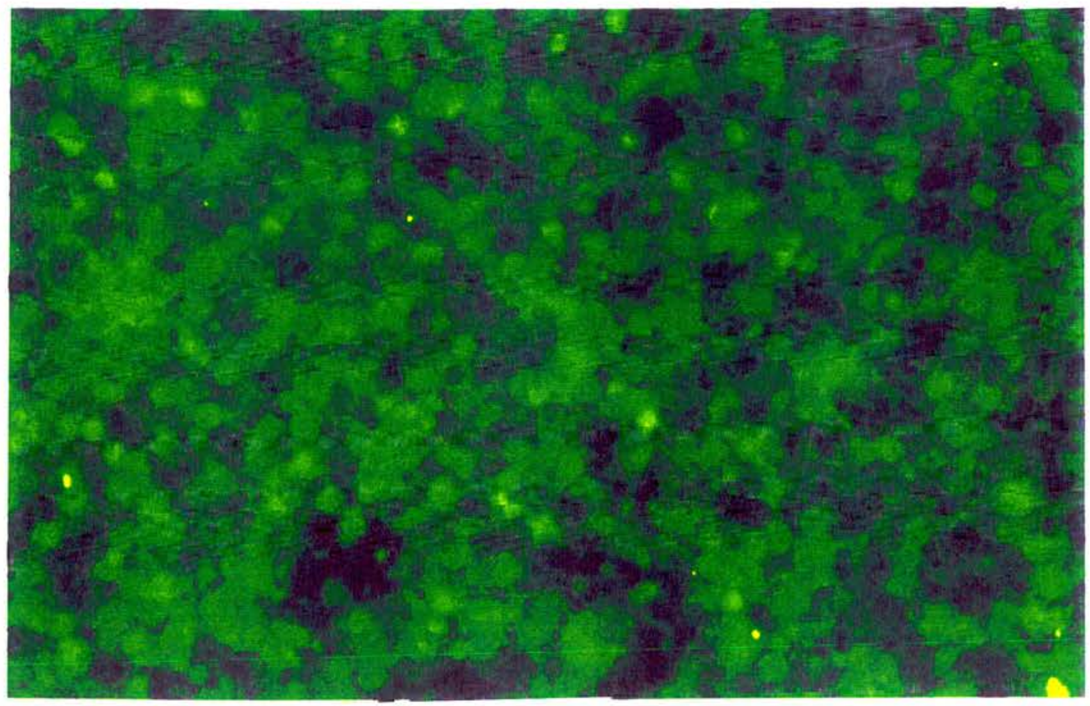
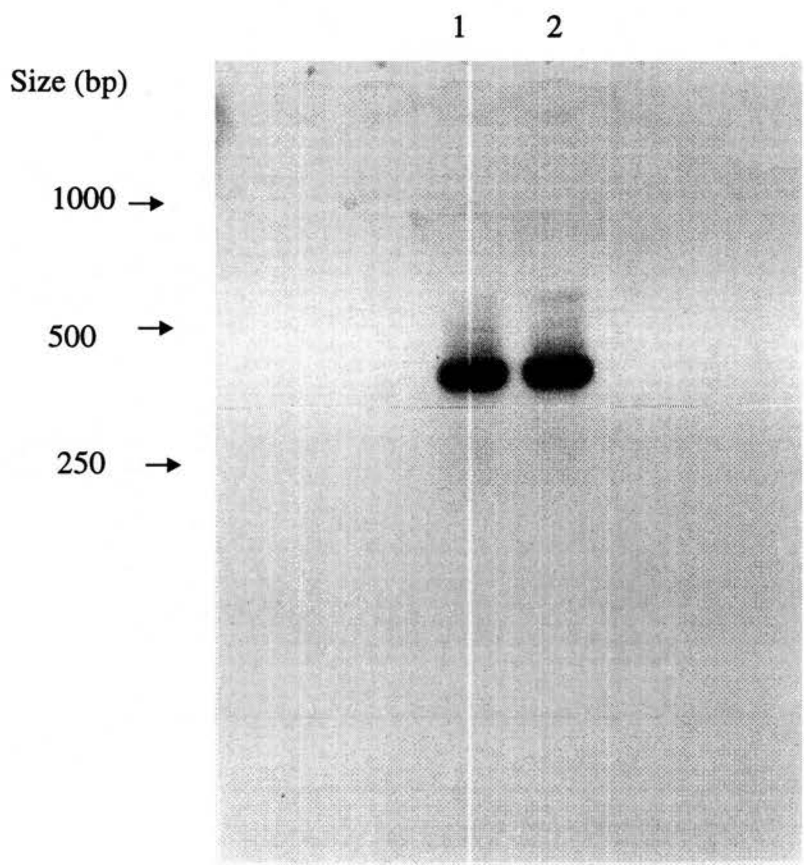


Figure 3.10. Diffuse cytoplasmic and nuclear fluorescence in OHV-2 LCL in an IIF using a P-5 antiserum (x 200).

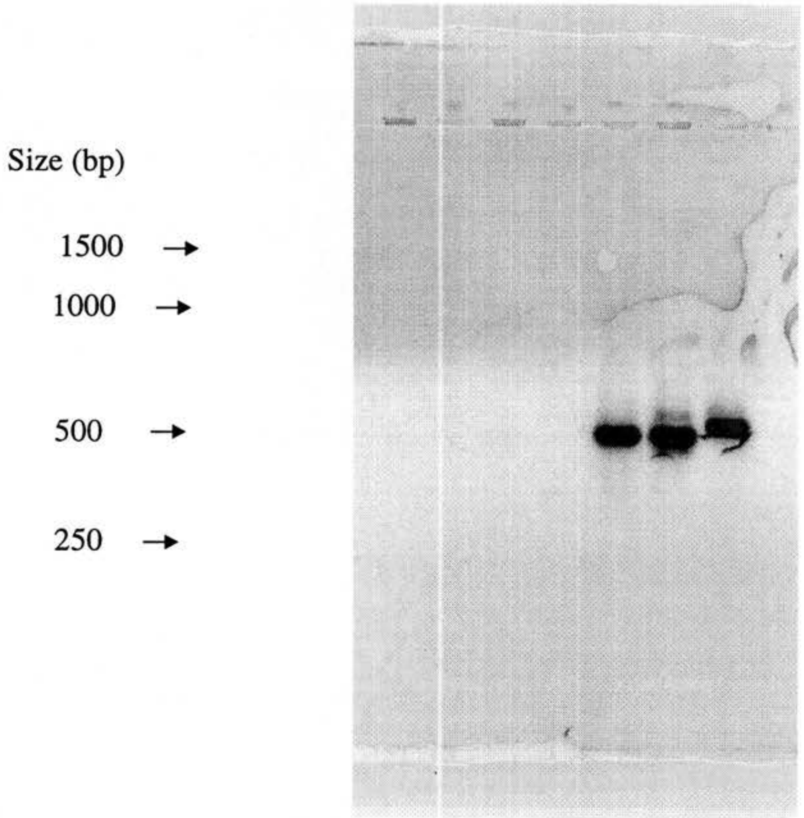
Figure 3.11-Southern blot of RT-PCR using P-1 primers and DIG labelled viral DNA PCR products as the probe.



Lane1-cDNA from RNA extracted from AHV-1 LCL

Lane2-cDNA extracted from RNA derived from OHV-2 LCL

Figure 3.12-Southern blot of RT-PCR using P-5 primers and DIG labelled viral DNA PCR products as the probe



Lane1and 3-cDNA from RNA extracted from AHV 1 and OHV-2 LCL using S21 and S20 primers

Lane2-cDNA extracted from RNA deived from AHV-1 LCL using primers S21 and T64

3.3.9 ELISA results

The antisera to P-5 were tested in triplicate against the antigen P-5 and against its fusion partner thioredoxin and the average OD readings recorded (tables 3.2 and 3.3). OD readings at the same serum dilution and at the same time post inoculation for the same rabbit, were consistently higher with the recombinant P-5 protein than with the fusion partner thioredoxin alone.

However, the results in table 3.4 indicate that the inoculated cattle did not develop antibody against polypeptide 5.

TABLE 3.2: RESULTS OF INDIRECT ELISA OF SERA OBTAINED FROM TWO RABBITS IMMUNISED WITH P-5 (values in O.D. at 492 nm) USING P-5 RECOMBINANT PROTEIN AS ANTIGEN).

	Rabbit no. 21			Rabbit no. 22		
SERUM	0*	25	60	0	25	60
1; 500X	0.231	2.071	2.377	0.114	3.126	3.177
1; 1000	0.051	1.674	2.150	0.043	2.887	2.000
1; 2000	0.088	1.396	2.215	0.056	2.712	2.215
1; 4000	0.034	1.041	2.024	0.074	1.954	1.75
1; 8000	0.057	0.842	1.667	0.072	1.243	1.662

* = Day post inoculation. X = Serum dilution

TABLE 3.3: RESULTS OF INDIRECT ELISA OF SERA OBTAINED FROM TWO RABBITS IMMUNISED WITH P-5 (values in O.D. at 492 nm) USING THIOREDOXIN AS ANTIGEN

	Rabbit no. 21			Rabbit no. 22		
SERUM	0*	25	60	0	25	60
1; 500X	0.263	1.390	1.379	0.184	1.400	2.060
1; 1000	0.081	0.888	1.050	0.113	0.902	1.238
1; 2000	0.089	0.789	0.694	0.106	0.722	1.046
1; 4000	0.047	0.641	0.602	0.074	1.539	0.458
1; 8000	0.042	0.342	0.408	0.172	0.243	0.366

* = Day post inoculation. X = Serum dilution

TABLE 3.4; P-5 INDIRECT ELISA RESULTS (values in O.D. at 492 nm) ON CATTLE INOCULATED WITH AHV-1 OR BHV-1.

Serum used at 1:50 dilution

	C500		WC11		BHV-1		Control	
D.P.I.[xx]	**G01***	**G02**	**G04**	**G06**	**G07**	**G10**	**G09**	**G08**
0	0.077	0.091	0.067	0.06	0.128	0.071	0.058	0.071
5	0.073	0.116	0.098	0.052	0.114	0.088	0.088	0.067
10	0.082	0.034	0.102	0.087	0.063	0.099	0.045	0.102
15	0.056	0.034	0.076	0.088	0.056	0.131	0.119	0.08
16	0.082	0.092	0.036	0.066	0.077	0.094	0.046	0.044
17	0.094	0.107	0.04	0.083	0.057	0.089	0.066	0.065
18	0.069	0.083	0.114	0.079	0.06	0.045	0.042	0.058

xx = Days post inoculation, * = Steer number.

3.4 DISCUSSION

The results show that the complete ORF of polypeptide 5 and part of the polypeptide 1 ORF were successfully cloned and expressed in *E coli.* Initial attempts to clone and express peptide 5 in pGEX were abandoned because of poor yield and reproducibility and because the product was insoluble (Dr. A. Rae, MRI, personal communication). pTRXfus was then chosen because thioredoxin is an *E. coli* protein which is highly soluble and also accumulates in the periplasmic space and is thus easily purified from the lysed cells. It was therefore assumed that this would help to make the recombinant protein soluble.

The expressed products have been used to raise polyclonal sera in rabbits and preliminary results from western blots using lysates from cells transformed with P-5 recombinants indicate that the immunisation was successful. Results using the P-5 fusion protein in an indirect ELISA show that the rabbit sera is reacting to both the fusion protein and the thioredoxin protein. However the reaction to the fusion protein is stronger than the reaction to Thioredoxin alone. Attempts to adsorb out the anti-thioredoxin activity by incubating the polyclonal sera with thioredoxin overnight at room temperature was unsuccessful. The ELISA results presented here show that though protein 5 is a strong immunogen the protein does not confer protection against virulent challenge. Rabbits immunised with P-5 reacted following challenge indicating that this protein is not protective.

The inability to detect mRNA specific for P-5 in cattle experimentally infected with AHV-1 indicates that this protein is not expressed in PBL *in vivo.* DNA extracted from the PBL from cattle infected with C500 during the clinical disease was however

AHV-1 PCR positive. P-5 antigen was not detected in PBL derived from cattle infected with either AHV-1 or BHV-1

The results could indicate that these proteins are not transcribed *in vivo*. The results may also suggest that very few cells express this protein *in vivo* and confirm results obtained by Edington and Patel (1981) who showed that AHV-1 antigens are expressed in $1/10^4$ of lymphocytes in experimentally infected rabbits.

Both P-1 and P-5 were detected in immortalised LCL, and also in cells infected with the virulent and the attenuated viruses, showing that both viral proteins are expressed *in vitro* by cells infected productively and non-productively. The presence of both antigens in AHV-1 and OHV-2 indicates that genes for P-1 and P-5 are conserved in both viruses.

The expression of P-5 in the attenuated virus was surprising in view of the reported truncation of P-5 in the attenuated genome (Handley et al, 1995). The clone ATT1 described by the authors was derived from CFA viral DNA.

During the course of this work, the complete nucleotide sequence of virulent AHV-1 has been published (Ensser et al, 1997). These authors have shown the sequences for P-1 and P-5 to be located in the middle of the 130 kbp L-DNA and identified P-1 as part of ORF50 which encodes an R Transactivator and P-5 as ORF A7 that encodes a C-type lectin..

The virulent virus (strain C500) that has been used in molecular studies in AHV-1 was isolated at least 24 years ago (Plowright et al, 1975). The virulent virus used to obtain the complete AHV-1 genome sequence was a cell free but virulent derivative of C500 which has undergone various passages in cell culture and also in cattle or rabbits since it was isolated in 1975. There is therefore need to obtain fresh isolates

of AHV-1 and use viral DNA from the wild-type isolates that have not undergone passage in cattle or culture to confirm the genome organisation in AHV-1.

CHAPTER 4

ISOLATION AND CHARACTERISATION OF WILD-TYPE ISOLATES OF AHV-1

4.1 INTRODUCTION

Characterisation of the AHV-1 genome (high passage attenuated, WC11 isolate) was initially conducted by Bridgen et al (1989). They showed that the genome comprises of approximately 130Kbp of unique DNA flanked by up to thirty 1kb tandem terminal repeats. Subsequently, the virus was classified as a gamma herpesvirus (Roizman et al, 1992). Handley et al (1995) cloned and sequenced DNA from the unique region adjacent the terminal repeats of the virulent C-500 isolate and its attenuated derivatives.The C500 isolate used in the study had been serially cloned using the limiting dilution method. The gene coding for one polypeptide, designated protein 5 (P-5) was found to be truncated in the attenuated virus. The authors suggested that this protein may be responsible for virulence. Another open reading frame, encoding putative protein 1 (P-1) was considered on structural grounds to be the equivalent of Herpesvirus saimiri transforming protein (STP-A11) (Beisinger et al, 1992). Ensser et al (1997) have published the complete sequence of the AHV-1 genome. The virus used was a cell free but virulent derivative of the C500 strain. The P-5 has now been identified as a putative protein encoded by an ORF, A7, with low homology to the family of cellular genes that code for C-type lectins. P-1 is part of a protein encoded by ORF50 which has a limitcd homology to the R-transactivators encoded by BRLF1 in Epstein Barr Virus (Hardwick et al, 1988), and ORF50 in Herpesvirus saimiri (Biesinger et al, 1992) (Ensser et al 1997). These proteins have now been shown to be encoded by sequences located in the middle of L-DNA in AHV-1 (Ensser et al, 1997) as indeed are their homologs in EBV

(Hardwick et al, 1988) and HVS (Nicholas et al, 1991). This is in contrast to the terminal location of these genes as reported by Handley et al, (1995).

Results of an RT-PCR using RNA extracted from AHV-1 and OHV-2 LCL showed that P-1 and P-5 are transcribed *in vitro* (Chapter 3). Results of indirect immunofluorescence on LCL immortalised by AHV-1 or OHV-2 and also on cell culture monolayers infected with AHV-1 indicate that these proteins are expressed *in vitro*.

The complete ORF of P-5 and part of the P-1 ORF were successfully cloned and expressed in *E coli* The expressed products were used to immunise rabbits but the rabbits succumbed to virulent challenge showing that immunisation against these proteins was not protective.

There is therefore a need to obtain fresh isolates of AHV-1 and determine the extent to which the putative genes for virulence are conserved between isolates. The aim of this work was to isolate and characterise fresh strains of AHV-1 from the natural host and cattle with MCF grazing close to wildebeest.

4.2 MATERIALS AND METHODS

4.2.1 History of wildbeest herds sampled

The wildebeest herds from which samples were obtained were located approximately 200 kilometres from the NVRC laboratories where isolation of virus was attempted. (Figure 4.1).These were resident herds in groups of approximately 50-300 which graze freely in an area outside the Maasai Mara game reserve called Aitong (Figure 4.1). The herds share common pasture with cattle since this land is owned by a Maasai Cattle Ranching group.

Permission to obtain samples was kindly granted by the Kenya Wildlife Services and shooting of the wildebeest was conducted by their staff. The cropping of wildebeest calves was conducted from April-July which was during and immediately after the calving season (Plowright, 1964). A maximum of four calves were shot during any one field trip. Samples were obtained from newborn calves upto 3 months of age. Newborn wildebeest calves have "brown" hair coat which is shed when they are three months old and the calves become more uniformly grey (Plowright, 1964) thus confidence in the age of the calves was assured..

Blood in EDTA, thyroid glands, kidneys and cornea were obtained asceptically from wildebeest the calves.

4.2.2 Preparation of cell cultures from Wildebeest

4.2.2.1 Trypsinisation cell culture

Wildebeest thyroid cells were prepared in an attempt to isolate AHV-1 which may be present in such tissues in a 'latent' form. The cells were prepared according to the method described by Plowright et al, (1960) with slight modifications. The tissues were washed with 3 changes of PBS and chopped into small (1-2mm) fragments using crossed scalpels and the fragments transferred into trypsinising flasks or transferred to cell culture flasks for explant culture. The tissue fragments transferred to trypsinising flasks were washed with 3 changes of PBS and then washed once with MEM (GIBCO). The supernatant was discarded and prewarmed trypsin (0.05% trypsin, 0.08% versene in Melnick buffer pH 7.4) added (approximately 100ml per gland or tissue) to the washed fragments. The contents were gently agitated with a

magnetic stirrer in an incubator at 37^0C. Trypsinisation was allowed to proceed for 30 minutes. The contents were centrifuged for 10 minutes at 400x g and the fragments resuspended in similar amount of trypsin. The cells were trypsinised for a further 1hr and the supernatant filtered through double muslin gauze. The supernatant was centrifuged at 800x g for 5 minutes and the cell pellet resuspended in an equal volume of MEM containing 10% ox serum and antibiotics (10μg streptomycin, 100U penicillin per ml). The cells were then counted using a haemocytometer and the concentration adjusted to 1-2 x 10^5cells/ ml. The cells were then seeded into cell culture flasks and incubated at 37^0 C. Cells were examined after 3 days when the media was changed to MEM + 5% ox serum + antibiotics. When the cell monolayer was confluent, cells were thereafter maintained with MEM containing 2% ox serum and antibiotics. Such cells were maintained for 14 days when they were passaged. To passage cell monolayers, media was decanted from culture flasks and the cells washed with prewarmed PBS and 10ml of prewarmed trypsin added. The trypsin was allowed to adsorb for 2 min and then discarded. The cultures were incubated for 10 min at 37^0 C and the loosely attached cells released from the flask surface by gentle pipetting with 5ml of ox serum. The cells were pelleted by centrifugation at 800xg for 5 min and the cells resuspended in 10ml of growth media. The cells were counted using a haemocytometer and the concentration adjusted to $2x10^5$ cells/ml before seeding into fresh culture flasks. The cells were then incubated at 37^0 C for the cells to form a monolaycr.

4.2.2.2 Explant culture of wildebeest tissues

To prepare explant cultures, approximately 1g of kidney or thyroid and whole cornea was chopped into small fragments using crossed scalpels in 5 ml of growth media and the chopped fragments placed in 25cm^2 cell culture flasks. The flasks were incubated at 37^0 C without agitation for 72 hours. The media was decanted and the visible tissue lumps loosely attached to the flask surface removed gently using sterile pipettes. The attached cells were gently overlaid with growth media cells incubated at 37^{0}C until they were confluent. The monolayers were thereafter maintained with MEM containing 2% ox serum and antibiotics. These primary cell cultures could be maintained for 14 days when they were passaged as described in 4.2.2.1.

Wildebeest cell cultures or explants that did not show cpe after 42 days were discarded.

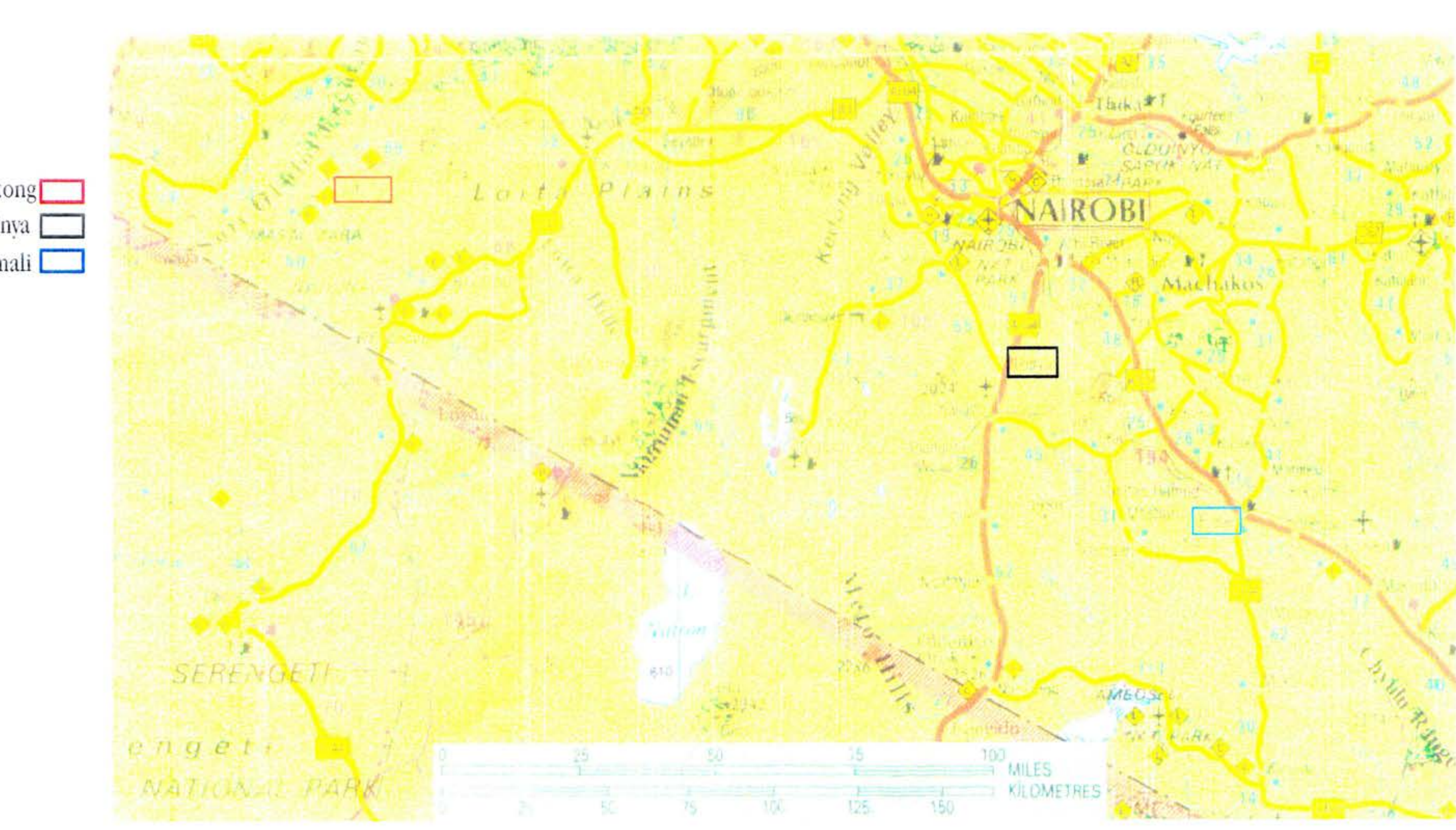
4.2.3 History of infected cattle sampled

Samples from clinical cases of MCF were obtained from cattle observed at a slaughter house located in Isinya in Kajiado district which is located approximately 200Km from the wildebeest herds used during the cropping exercise (Figure 4.1). The samples were therefore identified as Isinya 1 or Isinya 2 or Isinya 3 to denote the location and the animal number sampled. The animals were transported to the slaughterhouse from grazing ranches which were also inhabited by a resident group of approximately 1000 wildebeest composed of many herds. Normally the owners of the sick animals were identified with the assistance of the Veterinary staff at the

abattoir and the farms visited. Isinya 1 was an adult cow from a Zebu herd of about 600 animals of mixed ages which graze freely with the wildebeest. There was a small flock of local Red Maasai sheep. The farmer, Mr. Joseph Salaash informed us that the first case of MCF was observed in May 1997. The farm was visited on 18/8/97 and the owner informed us that he had sold 53 animals which he suspected to be MCF cases.

The other two cases obtained (Isinya 2 and Isinya 3) were from a ranch located near the town called Emali which is about 60 kilometers from Isinya. The ranch consists of approximately 1,200 Zebu cattle and 800 Black head somali sheep and Red maasai sheep and which graze freely together with wildebeest. One of the farmers, Mr. Paul Mokoa informed us that there are only sporadic cases but morbidity figures were not available.

Figure 4.1. The map shows the locations where samples for virus isolation were obtained. Locations indicated by coloured boxes.



4.2.4 Isolation of virus from cattle

Samples from clinical cases were collected on 13/8/97. The animals looked dejected, with stringy mucoid nasal discharge, enlargement of prescapular, precrural and submandibular lymph nodes and serous ocular and mucopurulent nasal discharge. Samples taken from clinical cases of MCF for virus isolation were blood in EDTA vacutainer tubes and prescapular lymph nodes. Approximately 20ml of EDTA blood was collected in vacuum tubes and approximately 5g of lymph node tissues were collected into MEM. All samples were transported to the laboratory on ice and processed for virus isolation on the same day.

Lymph node tissues were chopped into small fragments (1-2mm cubes) and resuspended in 20% (w/v) PBS. The fragments or EDTA blood were centrifuged at 800xg for 10 minutes. The buffy coat layer was collected and resuspended in an equal volume of PBS. The tissue fragments were also resuspended in equal volume of PBS. The buffy coat and tissue suspensions were then centrifuged at 800xg for 10 minutes. The pellets were resuspended in an equal volume of MEM and 2 ml inoculated into 25cm^2 secondary bovine thyroid cell cultures. The rest of the samples were stored at -70^{0}C in MEM containing 10% DMSO and 20% ox serum. The cells were allowed to adsorb for 2 hours at 37^0 C and the cells overlaid with 10 ml of maintenance media. The cells were incubated at 37^0 C for 48hr. The monolayers were washed 2x with PBS and the maintenance media added. The cultures were then observed daily for development of cpe for 14 days. The cultures were passaged (described in 4.2.2.1) and observed for a further 14 days. Cell cultures that did not show cpe were then discarded. Cells showing cpe were trypsinised and used for

extraction of DNA. Some of these cells were stored at -70^0 C in MEM containing 10% DMSO and 20% ox serum.

4.2.6 Extraction of DNA from tissue culture cells

Cell cultures showing cpe characteristic of AHV-1 were detached from culture flasks by trypsinisation and the cells pelleted by centrifugation at 800 x g for 10 minutes. The cells were washed twice with PBS and the cell concentration adjusted to 1 x 10^6 cells per ml. The cells were aliquoted into 1ml volumes and the cells pelleted using a microfuge at 10,000xg for 2 minutes. The supernatant was decanted, the pellet vortexed and 600μl of extraction buffer (150mM NaCl, 10mM Tris-HCl, pH 8.0, 10mM EDTA, 0.1% SDS) added to lyse the cells. The contents were mixed thoroughly and 600μl of phenol:chloroform (6:4) added to the mixture. The contents were centrifuged at 10,000x g using a microfuge for 10 minutes and 500μl of the upper aqueous layer removed. The aqueous layer was added to 500μl chloroform and the contents mixed vigorously for 2 minutes. The mixture was centrifuged again for 10 minutes at 10,000 x g using a microfuge and 400μl upper aqueous layer removed and 200μl of 7.5.M ammonium acetate added. To precipitate DNA, 800μl of ethanol was added to the contents and the mixture transferred to -20^{0}C for 2 hours. DNA was pelleted by spinning the contents in a microfuge at 10,000x g for 5 minutes. The DNA was resuspended in 100 μl of T.E. (pH 7.4). DNA was then used for PCR or stored at -20^0 C for further analyses.

4.2.7 Extraction of DNA from tissue samples

Approximately 0.5 g of tissue was chopped finely with a "criss-cross" action using scalpel blades and the tissues transferred to a 1.5ml centrifuge tube. The tissue was washed by addition of small volume of PBS and the contents centrifuged at 6000x g for 2 minutes in a microcentrifuge. The supernatant was discarded and the fragments washed again in PBS. The supernatant was discarded and 1 ml of Ammonium Chloride lysis buffer (0.17M Ammonium chloride, 0.17M EDTA, pH7.6) added to lyse any contaminating erythrocytes. The contents were centrifuged at 6000x g for 2 minutes using a microfuge and the supernatant discarded. 500μl of extraction buffer (150mM NaCl, 10mM Tris-HCl, 100mM EDTA, 0.1% SDS, 200μg Proteinase K/ml pH 8.0) was added to the tissue pellet. Digestion was carried out overnight at 37^0C or for 2hr at 55^0C. Proteinase K was then inactivated by boiling the contents for 5 minutes. The sample was used immediately for PCR or stored at -20^0C until required for PCR analysis.

4.2.8 Extraction of DNA from PBL

PBL was prepared by lysing 200μl whole blood obtained from cattle or wildebeest in Tris-EDTA buffer (0.1M Tris-HCl, 1mM EDTA, pH 7.6). DNA was extracted by incubating the PBL at 56^0C for 45 minutes in a buffer containing 50mM KCl, 20mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, 0.5% (v/v) Tween 20 to which was added 100mg/ml of freshly prepared proteinase K. The contents were boiled for 10 minutes to inactivate the protease before testing using PCR.

4.2.9 PCR

PCR was conducted to detect AHV-1 or OHV-2 sequences in DNA obtained from field cases of MCF. In all cases, AHV-1 and OHV-2 positive controls (kindly provided by Miss I. Pow, MRI) were included. AHV-1 PCR was conducted on DNA obtained from samples derived from cattle experimentally inoculated with fresh isolates of AHV-1.

4.2.9.1 PCR for detection of OHV-2 sequences

This reaction was conducted in two amplification cycles following the method described by Baxter et al, (1993) who amplified section of the tegument gene which corresponds to a position approximately 120kbp in the AHV-1 genome. In the primary reaction, primer sets used were;

556: 5'- AGTCTGGGTTATATGAATCCAGATGGCTCT-3',

and

755: 5'- AAGATAAGCACCAGTTAATGCATCTGATAAA-3'.

DNA was denatured by boiling for 3 minutes followed by 25 cycles at 94^0 C for 30 sec, 60^0 C for 30 sec, and 72^0 C for 30 sec.

The secondary reaction involved amplification of 5µl of the DNA amplified in the primary reaction using primer sets;

555: 5' -**TTCTGGGGTAGTGGCGAGCGAAGGCTTC**-3' and **556**.

Twenty five cycles of 94^0 C for 30 sec, 60^0 C for 30 sec, 72^0 C for 3 min were performed and one extension cycle at 72^0 C for 5 min. The PCR products were ran on agarose gels to confirm presence of fragment.

4.2.9.2 PCR for detection of AHV-1 sequences

Amplification of AHV-1 sequences was carried out using primers C317 and C318 primers as described in 3.2.2 (and see appendix 6.1). PCR products were analysed by electrophoresis on 1% agarose gel .

4.2.10 Virus neutralisation test

This test was conducted to screen cattle for AHV-1 antibodies before the animals could be used for inoculation with fresh isolates of AHV-1. The test was conducted as described in 3.2.21

4.2.11 Inoculation of cattle

Two steers, seronegative for AHV-1, were each inoculated with 2.5ml cell suspensions obtained from tissue culture flasks showing cpe after inoculation with samples from the field. This was approximately 1/4 of the cells obtained following trypsinisation of a $25cm^2$ tissue culture flask.

4.3 RESULTS

4.3.1 Isolation of AHV-1 and PCR

Ten primary thyroid cell cultures out of 28 thyroid glands were established using the trypsinisation technique. Four out of 10 thyroid cultures were established by explant culture. All these cultures were successfully maintained but 6 were lost following passage due to bacterial contamination. Corneal explant culture was attempted from six wildebeest calves but only four cell culture monolayers were successfully established. Ten kidney explant cultures were established but five were lost during passage due to bacterial contamination.

AHV-1 was isolated from 2 out of 28 wildebeest calves sampled. However tissue samples obtained from 16 of these calves were positive by AHV-1 PCR alone (Table 4.1 and 4.2). One isolate (WC8) was recovered from corneal explants. The other isolate was recovered from bovine thyroid cultures inoculated with buffy coat obtained from another wildebeest calf (WC28)

Two isolates were obtained from 2/3 Zebu cattle showing clinical MCF at Isinya. One isolate was derived from buffy coat (Isinya 1) and the other isolate was derived from prescapular lymph node (Isinya 2) in thyroid cell culture monolayers. No cpe was observed in cultures inoculated with buffy coats or lymph node cell suspensions derived from Isinya 3. EDTA blood and lymph node tissues from all three animals were however PCR positive for AHV-1 DNA. The size of the PCR products was 400bp in all cases. None of the samples obtained from the three animals was OHV-2 PCR positive.

The time taken to observe cpe in these cultures was 18 days for Isinya 1 and 20 days for Isinya 2. The cpe was characterised by one or two syncytia on the 25cm^2 flask. These foci of cpe did not spread to affect adjacent cells after further incubation. Cultures were harvested 3 days after appearance of cpe. Some of the infected cells were used to inoculate cattle. The rest of the cells were used for DNA extraction and PCR conducted using AHV-1 primers. All DNA samples were positive on PCR test.

Table 4.1; **AHV-1 PCR RESULTS CONDUCTED ON WILDEBEEST SAMPLES.**

CALF NO.	BLOOD	THYROID	KIDNEY	CORNEA
1	-VE	-VE	ND	ND
2	-VE	-VE	ND	ND
3	+VE	-VE	ND	ND
4	+VE	-VE	ND	ND
5	+VE	-VE	ND	ND
6	-VE	+VE	ND	ND
7	-VE	+VE	ND	ND
8	-VE	+VE	ND	ND
9	+VE	+VE	ND	ND
10	-VE	+VE	ND	ND
11	+VE	+VE	ND	ND
12	+VE	+VE	ND	ND
13	+VE	-VE	ND	ND
14	-VE	-VE	ND	ND
15	+VE	+VE	ND	ND
16	+VE	-VE	ND	ND
17	+VE	+VE	ND	ND
18	+VE	+VE	+VE	+VE
19	-VE	-VE	+VE	+VE
20	+VE	+VE	+VE	+VE
21	-VE	-VE	-VE	-VE
22	-VE	-VE	-VE	-VE
23	-VE	-VE	ND	ND
24	+VE	+VE	+VE	-VE
25	-VE	-VE	-VE	-VE
26	+VE	+VE	+VE	-VE
27	-VE	-VE	-VE	+VE
28	+VE	+VE	+VE	+VE

Indices; -ve = PCR positive +ve = PCR positive ND = Not Done

Table 4.2; SUMMARY OF RESULTS OF PCR CONDUCTED ON WILDEBEEST SAMPLES.

SAMPLE	NO. TESTED	NO. POSITIVE
EDTA BLOOD	28	16
THYROID	28	12
KIDNEY	10	5
CORNEA	10	6

Two isolates (WC28 and Isinya 2) were used to inoculate cattle. Steers inoculated intravenously with the WC28 isolate reacted 51 days after inoculation. One of the steers inoculated with the Isinya 2 isolate reacted 55 d.p.i. The other steer reacted 61 d.p.i. The disease was characterised by fever and nasal and ocular discharge. The animals were killed 3 days after appearance of clinical signs and tissues taken for PCR and virus isolation. PCR conducted on DNA extracted from prescapular and mesenteric lymph nodes from all the animals killed were positive for AHV-1. Virus isolation attempts were however unsuccessful due to bacterial contamination of cell cultures.

Post-mortem lesions observed in animals inoculated with either virus isolate were generally similar (Table 4.3) and compatible with a diagnosis of MCF.

TABLE 4.3; PATHOLOGY IN CATTLE INOCULATED WITH FRESH ISOLATES (WC28 OR ISINYA2) OF AHV-1)

ORGAN/ TISSUE	G20* (WC28)	G21 (WC28)	G22 (1sinya1)	G23 (Isinya 2)
Buccal mucosa	Congested	Congested	Congested	Congested
Nasal mucosa	Mucous, Petechiae	Mucous, Congested	Mucous, Petechiae	Mucous, Petechiae
Pharynx	NVL[xx]	Petechiae	Petechiae	Petechiae
Abomasum	NVL	NVL	Congested	Congested
Rectum	Petechiae	Petechiae	NVL	NVL
Liver	Mottled	NVL	NVL	Mottled
Kidney	NVL	NVL	NVL	Mottled
Larynx	NVL	Petechiae	Petechiae	NVL
Trachea	Frothy, Petechiae	Frothy	Frothy	Petechiae
Lung	Oedema	Oedema	NVL	Oedema
Heart	epicardial Petechiae	NVL	NVL	NVL
Spleen	Enlarged	Enlarged	Enlarged	Enlarged
Superficial L.node	Enlarged Moist, Petechiae	Enlarged Moist, Congested	Enlarged Moist, Petechiae	Enlarged Moist, Petechiae
Mesenteric L.node	Congested	Congested	Congested	NVL

* = Animal No. xx = No Visible Lesions

4.4 DISCUSSION

Two fresh isolates of AHV1 have been made from the natural host of the virus, and a further two from clinical bovine cases of MCF. Success rate in isolation of virus from affected cattle was high (2/3). The success rate in virus isolation from wildebeest calves reported in this work is low (2/28) although the number of wildebeest samples positive by PCR was high (16/28). Infection rate calculated from isolation of virus in blood obtained from wildebeest calves during the first 3 months of life has been reported to be 31% (Plowright, 1965b). The low success rate reported here was mainly due to contamination of cultures during the preparation of primary cultures or following passage of explant cultures or cell culture monolayers. In addition cpe due to AHV-1 is difficult to detect (Plowright, 1965a). Some inoculated cultures may have therefore been falsely recorded as negative and the cultures discarded due to inability to recognise cpe.

Two of the four isolates were experimentally inoculated into cattle and the cattle reacted. The cattle showed clinical disease typical of the "head and eye form" of MCF (Daubney and Hudson, 1936). Virus isolation attempts from experimentally infected cattle were unsuccessful due to problems of contamination of cell cultures. Blood and lymph node tissues obtained from these animals were however positive on PCR.

The wildebeest population from which two of the isolates were recovered could not have interacted with the clinically affected cattle as the wildebeest populations were resident of an area located approximately 200km from where the sick cattle were observed.

The prevalence of the disease could not be established during the period of study. This was mainly because the exercise to isolate virus from wildebeest calves (April-July) coincided with the period when the disease incidence in cattle is high (Plowright et al, 1960, 1965b). AHV1 genomic studies have depended on two virus strains isolated in Kenya many years ago (Plowright et al, 1960, 1975). There is no recent data available on the incidence or prevalence of the disease but a study of the economic impact of the disease in Kajiado district and Narok districts of Kenya is being conducted (Dr P. Mirangi-personal communication). Plowright et al (1975) reported that in 1970, the incidence rate in both districts was 7%.

Ensser et al, (1997) have described the complete nucleotide sequence of the C500 isolate of AHV1 and they have shown the sequences for P-1 and P-5 to be located between 73-79kbp on the 130kbp. The availability of DNA obtained from fresh AHV-1 isolates provides an opportunity to map the genes of these two proteins and determine the extent to which these putative determinants of virulence are conserved between isolates. This is the aim of the next chapter.

CHAPTER 5

COMPARISONS BETWEEN GENOMIC DNA DERIVED FROM WILDTYPE, LOW AND HIGH PASSAGE C500 AND ATTENUATED WC11 STRAINS OF AHV-1

5.1 INTRODUCTION

Studies of AHV-1 have largely been with one of two isolates. Isolate WC11 was recovered from wildebeest (Plowright, 1965b) and following many passages in tissue culture is non-pathogenic for cattle. The C500 isolate was recovered from an ox reacting with MCF (Plowright et al, 1975) and exists in two forms. The virulent form is largely cell-associated though low tissue culture passage cell free virus is also pathogenic for rabbits (Russel , 1980). Following additional passage however, the virus no longer causes MCF following inoculation into rabbits. The genomes of these C500 derivatives were compared by restriction enzyme analysis (Handley et al, 1995). Differences in Sma 1 profiles of the C500 forms were described and three restriction fragments designated vir 1, vir 2 and att 1 were cloned and sequenced. The clones were mapped to a terminal location in the viral genome based on their hybridisation to the ladder of restriction fragments which results from the variable number of tandem repeat units at each end of the unique DNA. Differences in the nucleotide sequence of parts of these clones were interpreted to be due to deletions which occurred on rearrangement of the genome during the transition from virulence to attenuation. ORFs 1 and 5 were described which because of limited structural homology and their position in the genome adjacent to the terminal repeats, were considered to be AHV-1 homologues of the STP and TIP genes of HVS (Murphy et al, 1989, Beisinger et al, 1990). Furthermore, the differences in the sequences of the clones from the attenuated and virulent viruses indicated that some of these ORFs were altered in the change from virulence to attenuation and were therefore of importance to the viral pathogenesis.

The publication during this study of the complete sequence of the AHV-1 genome (Ensser at al, 1997) has necessitated a reassessment of the Handley et al (1995) clones for two reasons. Firstly, there were differences in detail between the two sequences. Secondly, while the sequence of the vir2 clone of Handley et al (1995) came entirely from the middle of the genome around 75kbp, the vir1 clone contains sequence from the middle of the genome linked to sequence from the 3' end. This raised the question as to whether vir1 was an artefact of the cloning procedure.

The first objective of this chapter was to establish whether the sequence differences were real polymorphisms between the two viral genomes which were sequenced or were they sequencing errors. The second objective was to use PCR and sequencing to prove the presence of the rearranged clone in the viral genome we were dealing with which was not observed by Ensser et al (1997).

Re-sequencing has allowed the identification of ORFs in the Handley et al (1995) clones which have now been shown to have homologues in other gammaherpesviruses. This will be described here.

With the availability of new virulent isolates of AHV-1, a third objective was the sequencing of those ORFs in the block of DNA around 75kbp which are involved in the rearrangement to determine whether or not there are any differences between isolates in this interesting region

5.2 MATERIALS AND METHODS

5.2.1 Target viral DNA used in PCR.

(1) DNA extracted from 4 fresh isolates of AHV-1 (CHAPTER 4) i.e. Isinya 1, Isinya 2, WC 8 and WC 28.

(2) Low passage C500 isolate (Plowright et al, 1975) biologically cloned from an infected rabbit by three sequential passages by limiting dilution in bovine turbinate (BT) cell cultures. The infectivity was largely cell associated (CA). DNA from this derivative designated CA.

(3) Intermediate passage (I.P.) PP that was cell free. DNA extracted from this derivative was designated I.P.

(4). CA that had been attenuated after 30 serial passages in BT. Infectivity was largely cell free. This derivative was designated CFA.

(5) CA that had been serially passaged 464 times in BT. The virus was mainly cell free. This derivative of C500 was designated high passage (HP).

5.2.2 PCR and cloning

PCR was conducted to establish the presence of specific DNA fragments in the various AHV-1 DNA samples and also to obtain products for sequencing. The sequences of the oligonucleotide primers were taken from the published C500 genome (Ensser et al, 1997) are given in table 5.1. The respective positions of the primers on the genome are given for each primer.

PCR products were cloned into either pGEM-T or pBluescript (KS$^-$)(described in section 2.4). Amplification used *Taq* or polymerase mix (described under section 2.5.15). *Taq* adds an extra Adenine diphosphate at the 3'of the PCR product. Such products can therefore be cloned directly into pGEM-T. Amplification using the polymerase mix protocol has the advantage in that this method can be used to amplify longer fragments of DNA and without significant sequencing error.

Table 5.1; Primers used to conduct PCR for genomic comparisons. Position of the primers in the AHV-1 genome are shown in appendix 6.1

Primer	Sequence	Position in genome	R.E[0]
V5582	5'-acactgtcgactatggctatggtctggtca-3'	73600-73571	Sal I
V7335(R)	5'-gaaccaggatccaatgtttagcggaggga-3'	74919-74890	Bam HI
Z4878	5'-tggctggatcctgagatgttggcagaaatg-3'	76554-76583	Bam HI
Z4849(R)	5'-gctagtcgacagttatccatggacgggtgc-3'	77283-77312	Sal I
14	5'-ataaccttcaacagcacc-3'	77431-77448	
14(R)	5'-tcatccccagtaaaagta-3'	78971-78989	
V0144(R)	5'-aaccttcttggcggcattcatcat- 3'	73608 -73631	
Y0332(R)	5-gca ggcgccacttggtctgttggttagatg-3'	72601-72630	
V7822	5'-tctttgggatcccatgcataagcactctgc-3'	75213-75242	
P7	5'-gggcttgctgggtcgtagt-3'	129004-129028	
LUCS1	5'-ttgtgactcgagttaaaaaaaagtaggtg-3'	72664-72692	Xho I
LUCS2	5'-catactctcgaggttttctggatacaccc-3'	72356-72384	Xho I
LUCRR	5'-gttggcagatcttttgggtagtcaaattt-3'	72808-72836	Bgl II
1040(R)	5'-tgctgatggtcgtttatgtgctgt-3'	1040-1064	
1991(R)	5'-gacatggtctgggaataacactac-3'	1991-2014	
3153(R)	5'-gttgtgagataggtctacatgtcc-3'	3153-3177	
RP2	5'cgggtgctcagcctcacaga- 3'	1083-1102*	
RP1	5'-tctgtgaggctgagcacccg-3'	30-50*	

[0] =The enzyme restriction sites. Sites on the sequence are underlined. *= Sequences from L DNA

5.2.2.1 PCR to amplify a region within ORF 50 and sequences immediately upstream of ORF 50

This PCR was performed to prove the terminal location of the clones vir 1 and att 1 (Handley et al, 1995). The amplification reaction was conducted using *Taq* PCR. The PCR was also used to determine the extent to which the genomic rearrangements occur during attenuation of the virus with respect to the ORF50 and its promoter.

The reaction was conducted using primer RP1 which corresponded to a sequence from the terminal repeat unit at position 50-30bp on the H-DNA relative to the first Sac I site, together with primer V0144 (Table 5.1) on the complementary strand.

Primer V0144 was also used as a downstream primer together with primerY0332 (table 5.1 and appendix 6.1) to determine whether there has been a translocation or a translocation/duplication as reported by Handley (1993).

To prove that clone vir 1 was not a cloning artefact, a PCR was conducted using primers from the middle of the genome and primers designed from the 3'end of L-DNA. This reaction was performed on viral DNA obtained from wild-type virus, low passage, intermediate passage and CFA C500, and WC11 with primers V7822 and P7 (see table 5.1 and appendix 6.1)

This product was cloned into pGEMT and sequenced.

5.2.2.2 Primers used to amplify viral DNA sequences at the left end of the genome adjacent the terminal repeat

A primer from the terminal repeat, RP2 (table 5.1) was used in conjuction with reverse primers from within the left end of the L-DNA to try to obtain information

about the extent to which a translocation to the left hand end of the L-DNA had occurred. The reverse primers were at positions, 1040, 1991 and 3153 and their sequences are given in table 5.1 and their position in the AHV-1 is shown in appendix 6.1.

RP2 corresponds to the sequences 31to 50 relative to the end of the first terminal repeat adjacent the 5' end of the unique DNA sequences (Handley et al, 1995).

Annealing temperature for the four primers was 51^0C.

5.2.2.3 PCR to amplify a region immediately upstream of ORF50

These primers were initially constructed to clone the putative promoter for ORF 50 for assay of promoter activity *in vitro* (Chapter 6). The target DNA was from CA and CFA virus and DNA obtained from the fresh isolates of AHV-1. PCR was conducted with primers LUCS1 and LUCS2 as upstream primers in conjunction with LUCRR which was a 3' primer situated at start of exon 1 of R transactivator (table 5.1 and appendix 6.1).

5.2.2.4 Cloning and sequencing of ORF 50

Target DNA was genomic DNA from field isolates of AHV-1. This fragment was amplified using the Long Template PCR with primer V5582 and V7335 (table 5.1), annealing temperature of 57^0 C. The PCR products (Figure 5.1) were extracted from agarose gels, enzyme restricted using Sal I and Bam HI and cloned into pBluescript (KS^-) which had been digested with the same enzymes. Ligation mixtures were used to transform competent JM109 cells and clones containing the recombinant plasmid

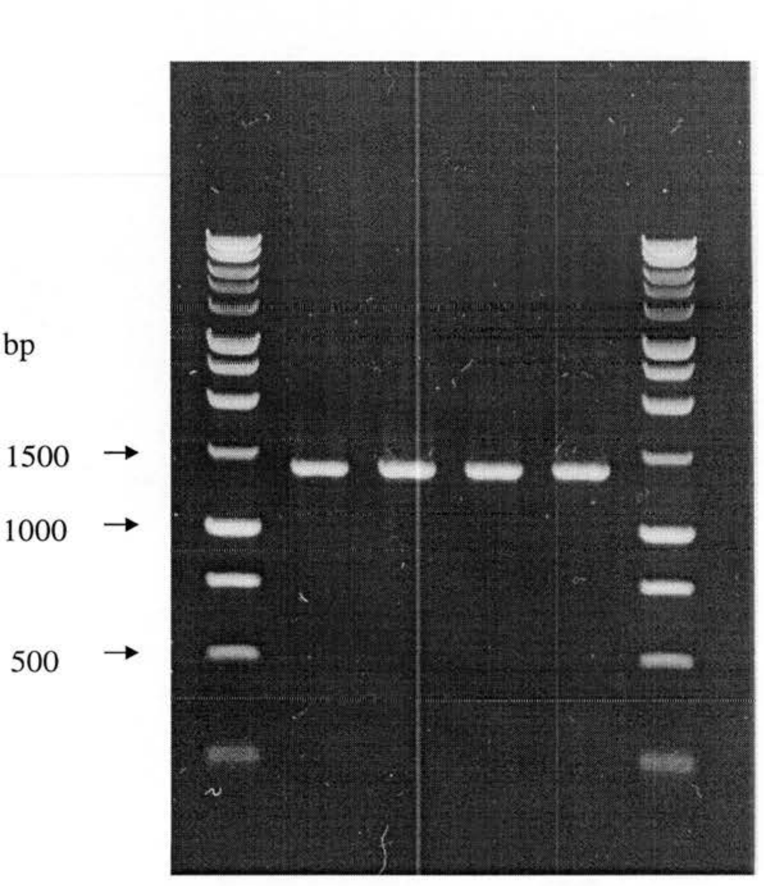
were confirmed following digestion with Sal I and Bam HI. DNA concentration was adjusted to 200ng/μl for sequencing.

5.2.2.5 Cloning and sequencing of ORF A7 (peptide 5)

PCR was performed using *Taq* polymerase to generate fragments of ORF A7 from genomic DNA obtained from field isolates for sequencing. Annealing temperature was 60^0C. PCR was conducted using primers Z4878 and Z4879 (Table 5.1 and appendix 6.1)

The PCR products were cloned into pGEM-T and the recombinant plasmids used to transform JM109 cells. Miniprep DNA was prepared and then digested with Sal I and Bam HI to confirm presence of insert in the recombinants. ssDNA was prepared from clones constructed using viral DNA from wild-type virus. ds DNA was prepared from CFA for sequencing.

Figure 5.1 ; PCR using DNA from the four wild-type isolates and ORF50 primers



Lane1 and 6- Molecular wt. markers

Lane 2-5- PCR results on the four new isolates

5.2.2.6 Cloning and sequencing of ORF A8 .

This ORF is predicted to be 1559bp in length (Ensser et al, 1997). *Taq* PCR was conducted to determine whether the A8 PCR fragment was present in the wild-type virus, high passage C500 and also in the attenuated WC11. Primers 14 and 14 (R)(Table 5.1 and appendix 6.1) were constructed to amplify full length A8, the ORF which encodes the putative virus attachment protein.

5.2.3 Nucleotide analysis and amino acid analysis.

Nucleotide analysis was conducted as described in 2.5.10. Translation of nucleotide sequence to amino acid sequences was done using the programs of UWGCG package. Potential coding regions were identified using TESTCODE (Fickett, 1982). Searches for amino acid homologies were conducted using PROSRCH programme (Lipman and Pearson, 1985)

5.3 RESULTS

5.3.1 Location of peptide 1 in the attenuated genome

The sequence of the PCR product amplified using the RP1 primer from the tandem repeats in conjunction with the gene-specific primer for peptide 1 is shown in figure 5.2 together with the translation of peptide 1 up to the location of the second primer. The first 288 bp of sequence, shown in capitals in figure 5.2, are > 92% homologous

to the sequence of the tandem repeats from position 50 back through the Sac I site to position 867 bp of another repeat unit. The rest of the clone is 100% homologous to the unique DNA starting at 72673 bp and includes the gene for peptide 1 which is normally found at position 72825-74903. The transcriptional direction of peptide 1 in this PCR product is away from the sequence of the tandem repeat. Because of the orientation of the RP1 in the tandem repeats, this implies that this clone comes from the right hand or 3'end of the L-DNA.

PCR reactions using primers Y0332 from 72601(i.e. upstream of peptide 1 ORF) (see appendix 6.1) and V0144 from position 73631(which is within the P-1 ORF) were positive for only the wild-type and CA C500 isolates amplifying a fragment of 1000bp as expected from the AHV-1 sequence (see figure 5.3)

Figure 5.2 Sequence of the PCR fragment obtained from CFA C500 using primer RP1 from the terminal repeats and a primer V0144 from position 73631bp.

The figure shows the sequence and aminoacid acid alignment for the PCR fragment. Details are explained in 5.3.1

The repeat sequence is in capitals, primers and informative restriction sites are in bold face and intron/exon boundaries are underlined (Ensser et al. 1997).

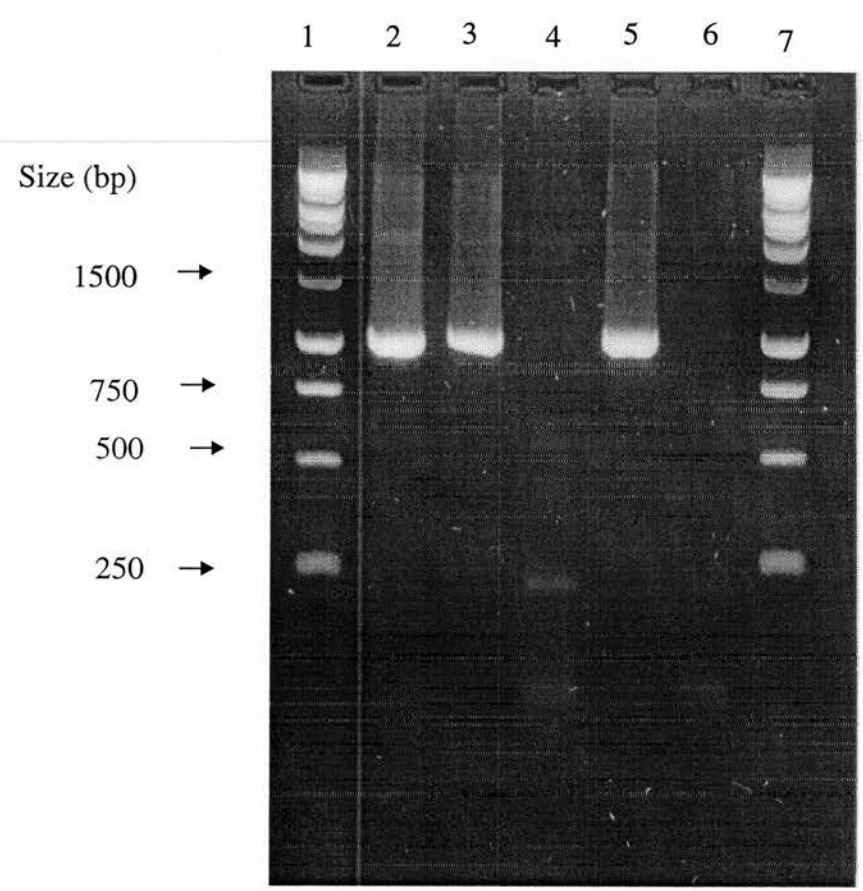
```
RP1                                             Sacl
TCTGTGAGGCTGAGCACCCGAGTCAACCCTAGGGGAGCCGGCTGAGCTCTGTTGGGCCCA   60
GAGACCCGGAGAGAGGGGAAAAAAAACCAGGGGGACGGCCGCGGGGGGCTCGGGGGGCG   120
GGGAGGGCCGCGGACGACAAAAGCCCCGGGGCGACCCGGAAAGGGGCGTGGCGCTCCCGC   180
CAGAGAGGTCCCGAGATCTCGGGACCTGCCACGCCCCCCTGCCGGCAAAAAAATGGGGCC   240
AGGGGGCGGGGAGGGCCGCGGAGAAAAAAACCTGGGGGCCGGGCGATGCaagttaaaaaa   300
aagtaggtgacaggcttaaataacaagccacaggaactgtactcactaaatctgaccaga   360
ttgtttaagcagccagtgagtaagctcacatttatttctctaaggcttcatttcacaggc   420
                      M  S  A  N  N  P  S  C  A  S  R  D  P
acacaaatttgactacccaaaatgagtgccaacaacccctcatgtgcatccagagatcct   480
 P  P  K  K  V  R  T  F  F  S  S  I  R
cctcctaaaaaggtaaggactttttttttcttctatcaggtcatttttaaatggagactta   540
```

```
    Sma1
tcttcccgggaaacagtaaaccacatgtgttggacttaaagtttgcttgatctaattact   600
tgattatgtaacagccttttatactaacatatttattttaggatgattaataataaaca   660
atgactatcctaaaaatgtttttgcatactttaaggtttaaaaatctaaattatattttt   720
                               R  L  S  R  P  I  C  I  D  D  F  I  D  I
taaatcatttacagaggagactatcaagaccaatatgcatagatgacttcatagacatc   780
 T  A  D  L  G  D  T  I  G  A  A  L  K  S  F  Q  Q  N  N  A
actgctgatcttggtgatacaataggggctgctttaaagtccttccagcaaaataatgct   840
 C  T  Q  E  Q  S  E  Q  F  T  R  E  V  Y  D  V  C  K  N  I
tgcactcaagaacagagtgagcagtttaccagggaGgtatatgatgtctgcaaaAatatt   900
 L  Q  E  N  K  F  R  N  E  M  F  G  F  V  A  D  M  N  L  L
Ctccaggagaacaaatttagaaatgaaatgtttggatttgttgctgatatgaacctgcta   960
 N  L  F  A  L  F  R  S  Y  K  Q  R  V  R  T  H  F  G  K  Q
AatctatttgccctgttcagaagctataagcagagagttaggaccCactttggaaaacag   1020
 L  L  C  A  T  A  S  S  Q  I  I  R  F  F  L  E  R  V  I  R
CTtctatgtgccactgcctcatctcaaataattagattcttttagaaagagttataagg   1080
 H  T  D  K  W  F  L  L  A  P  C  N  G  L  I  L  P  Q  E  L
ctcActgacaaatggttttgttagcccctgcaatggcctcattctcccacaagagctg   1140
 A  K  E  M  Y  V  L  L  S  E  A  R  G  K  A  L  N  Q  G  R
                                              Hind111
gcGaaggaaatgtatgttctcctgtccgaagccaggggaaagctttgaaccaagggaGa   1200
                                V0144
 M  F  S  G  G  R  Q  N  M  M  N  A  A  K  K  V  L  T  V  Y
atgtttagcggagggagacaaaacatgatgaatgccgccaagaaggtt
 S  S  L  R  D  D  G  E  I  S  P  E  V  K  A  Y
```

5.3.2 Validation of the vir 1 clone and its location at the 3'end of the genome

When the primers located at positions 75213 (V7822) and 129004 bp (P7) (appendix 6.1) in the virulent cell free derivative of the C500 isolate were used to amplify DNA from the low passage derivative of C500 (CA), new virulent isolates and the CFA preparations, only the CFA DNA was positive. The sequence of this PCR fragment is shown in figure 5.4. Comparison with the published sequence shows that a block of DNA from position 75481 bp to position 72673 (see section above), has been translocated and placed between position 129840 bp of the unique DNA and the terminal repeat position in an inverted orientation. In this position the promoter region of the R transactivator has been truncated and this will be discussed further in chapter 6. Furthermore, ORF A10 (at position 130229-128811bp) which encodes a putative glycoprotein (Ensser et al, 1997) is interrupted by this translocation as is ORF A6 (72226-75858), a potential transactivator. The genomic rearrangement that occurs following attenuation of AHV-1 is summarised in figure 5.5

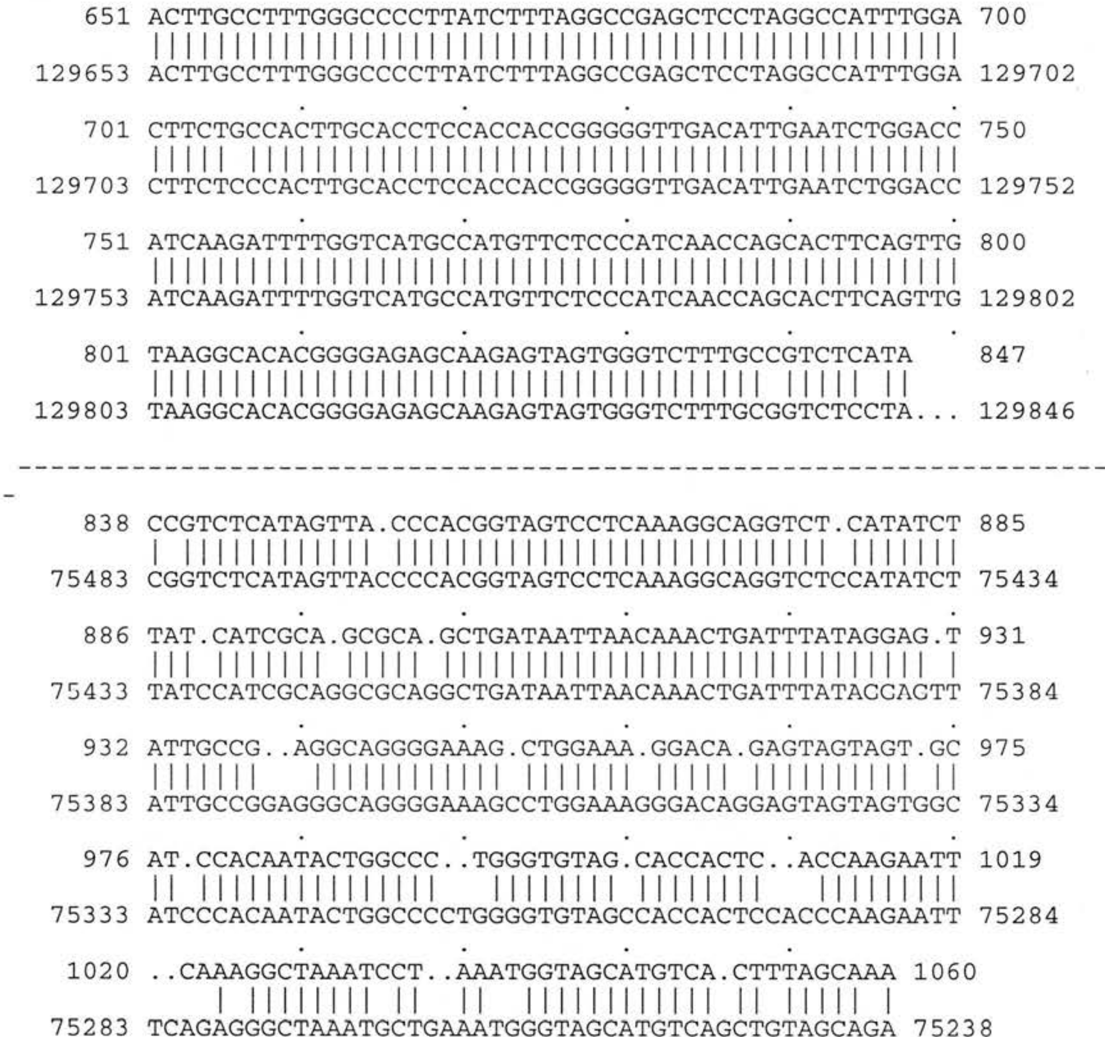
Figure 5.3 ; PCR of the wild-type and attenuated C500 isolates using primers V0332 and Y0144



Lane 1 and 7= Molecular Wt. Markers
Lane 2= Low passage C500 (CFA) DNA
Lane 3= Isinya 1 DNA
Lane 4= High passage C500 (CFA) DNA
Lane 5= CA (C500) DNA
Lane 6= WC11 (CFA) DNA

Figure 5.4. BESTFIT of the sequence of the PCR fragment obtained from AHV1 CFA C500 target DNA using primers P7 and V7822 (see Appendix 6.1) compared with the AHV1 sequence of Ensser et al 1997. Note the breakpoint in the sequence at 129846 bp where sequence from the middle of the genome at 75483 bp has been inserted.

```
       Primer 7
     2 GGGCTTGCTGGGTTCGTAGTCCCTAGGTGGTAAAGGAGGAGGTTCTTGCC 51
       ||||||||||| ||||||||||||||||||||||||||||||||||||||
129004 GGGCTTGCTGG TCGTAGTCCCTAGGTGGTAAAGGAGGAGGTTCTTGCC 129052
                .         .         .         .         .
    52 TGCCTCTACCTGCAGGAGCTTTGCTACGTGGAGTAGCCCCACTCAGACCA 101
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129053 TGCCTCTACCTGCAGGAGCTTTGCTACGTGGAGTAGCCCCACTCAGACCA 129102
                .         .         .         .         .
   102 CTGCTCGTAGATTCCTTGCTCTGTGGAGTAGGACCACGCTGACCACTTCC 151
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129103 CTGCTCGTAGATTCCTTGCTCTGTGGAGTAGGACCACGCTGACCACTTCC 129152
                .         .         .         .         .
   152 TGCAGGATCTCTGCTTGGAAGAGGGGGCTTTTTAGGACCACCTCCTGCAG 201
       |||||||||||||||||||||||| |||||||||||||||||||||||||
129153 TGCAGGATCTCTGCTTGGAAGAGGAGGCTTTTTAGGACCACCTCCTGCAG 129202
                .         .         .         .         .
   202 CACCTTTGCGGTCATCCAGGTCATCATCTGATATAGTTAGGTATACAGGA 251
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129203 CACCTTTGCGGTCATCCAGGTCATCATCTGATATAGTTAGGTATACAGGA 129252
                .         .         .         .         .
   252 GTTACAGGAGTTCCAATCGTTTTCACTTTAGTATTTCGATGTATTCCTTT 301
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129253 GTTACAGGAGTTCCAATCGTTTTCACTTTAGTATTTCGATGTATTCCTTT 129302
                .         .         .         .         .
   302 CCTTCGGTCCCATGGGGGCGTTGAACCACTAGATAGCAAGACCGCACAGG 351
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129303 CCTTCGGTCCCATGGGGGCGTTGAACCACTAGATAGCAAGACCGCACAGG 129352
                .         .         .         .         .
   352 GGTCCCATAAGGGTGTAGGGCATGGTGGCTTCGTTCCTGGAGGCAGCCAC 401
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129353 GGTCCCATAAGGGTGTAGGGCATGGTGGCTTCGTTCCTGGAGGCAGCCAC 129402
                .         .         .         .         .
   402 ATTTTTTTGTACGGATTCTCATCACCAGGAGCAGCTTCAATGACATCATG 451
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129403 ATTTTTTTGTACGGATTCTCATCACCAGGAGCAGCTTCAATGACATCATG 129452
                .         .         .         .         .
   452 CCTACTTGGGTTGGTATCCACCGGACCAGGCCCTGGAAATGCTAAAGGAA 501
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129453 CCTACTTGGGTTGGTATCCACCGGACCAGGCCCTGGAAATGCTAAAGGAA 129502
                .         .         .         .         .
   502 CTTTTTCTGGACGAGCTCCTGGGTCTTTTGGTGGGTTCTTGGCTGCAGG. 550
       |||||||||||||||||||||||||||||||||||||||||||||||||
129503 CTTTTTCTGGACGAGCTCCTGGGTCTTTTGGTGGGTTCTTGGCTGCAGGC 129552
                .         .         .         .         .
   551 CCGTCTTTAGGTGGGGACTTGCCTTTGGGCCCCTTCTCCTTAGGCCGAGC 600
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129553 CCGTCTTTAGGTGGGGACTTGCCTTTGGGCCCCTTCTCCTTAGGCCGAGC 129602
                .         .         .         .         .
   601 CCCTAGGTCCTTGGGCGGGGTCTTGCCTGCAGGCCCGCATTTAGGTGGGG 650
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129603 CCCTAGGTCCTTGGGCGGGGTCTTGCCTGCAGGCCCGCATTTAGGTGGGG 129652
                .         .         .         .         .
```



```
   651 ACTTGCCTTTGGGCCCCTTATCTTTAGGCCGAGCTCCTAGGCCATTTGGA 700
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129653 ACTTGCCTTTGGGCCCCTTATCTTTAGGCCGAGCTCCTAGGCCATTTGGA 129702
                 .         .         .         .         .
   701 CTTCTGCCACTTGCACCTCCACCACCGGGGGTTGACATTGAATCTGGACC 750
       ||||| ||||||||||||||||||||||||||||||||||||||||||||
129703 CTTCTCCCACTTGCACCTCCACCACCGGGGGTTGACATTGAATCTGGACC 129752
                 .         .         .         .         .
   751 ATCAAGATTTTGGTCATGCCATGTTCTCCCATCAACCAGCACTTCAGTTG 800
       ||||||||||||||||||||||||||||||||||||||||||||||||||
129753 ATCAAGATTTTGGTCATGCCATGTTCTCCCATCAACCAGCACTTCAGTTG 129802
                 .         .         .         .         .
   801 TAAGGCACACGGGGAGAGCAAGAGTAGTGGGTCTTTGCCGTCTCATA    847
       |||||||||||||||||||||||||||||||||||||| ||||| ||
129803 TAAGGCACACGGGGAGAGCAAGAGTAGTGGGTCTTTGCGGTCTCCTA... 129846

----------------------------------------------------------------
-
   838 CCGTCTCATAGTTA.CCCACGGTAGTCCTCAAAGGCAGGTCT.CATATCT 885
       | |||||||||||| ||||||||||||||||||||||||||| |||||||
 75483 CGGTCTCATAGTTACCCCACGGTAGTCCTCAAAGGCAGGTCTCCATATCT 75434
                 .         .         .         .         .
   886 TAT.CATCGCA.GCGCA.GCTGATAATTAACAAACTGATTTATAGGAG.T 931
       ||| ||||||| ||||| ||||||||||||||||||||||||||  |  |
 75433 TATCCATCGCAGGCGCAGGCTGATAATTAACAAACTGATTTATACCACTT 75384
                 .         .         .         .         .
   932 ATTGCCG..AGGCAGGGGAAAG.CTGGAAA.GGACA.GAGTAGTAGT.GC 975
       |||||||   |||||||||||| ||||||| ||||| |||||||||| ||
 75383 ATTGCCGGAGGGCAGGGGAAAGCCTGGAAAGGGACAGGAGTAGTAGTGGC 75334
                 .         .         .         .         .
   976 AT.CCACAATACTGGCCC..TGGGTGTAG.CACCACTC..ACCAAGAATT 1019
       || |||||||||||||||   |||||||| ||||||||   |||||||||
 75333 ATCCCACAATACTGGCCCCTGGGGTGTAGCCACCACTCCACCCAAGAATT 75284
                 .         .         .         .
  1020 ..CAAAGGCTAAATCCT..AAATGGTAGCATGTCA.CTTTAGCAAA 1060
         | ||||||||  ||  ||  ||||||||||||| || ||||| |
 75283 TCAGAGGGCTAAATGCTGAAATGGGTAGCATGTCAGCTGTAGCAGA 75238
```

5.3.3 Comparison of the predicted amino acid sequences of peptide-1 and peptide-5 with sequences in the SwissProt database.

Ensser et al (1997) have designated the ORFs corresponding to peptide 1 and peptide 5 ORF50 and A7 respectively. In SwissProt database searches for similarities to ORF 50, the best matches were to three gammaherpesvirus R transactivators, namely, from EBV, an immediate early (IE) gene ORF BRLF1 (Baer et al, 1984), from HVS, an IE, R encoded by ORF 50 (Nicholas et al, 1991) and from BHV-4 an IE gene IE-2

(Van Santen, 1993). The percentage identities and similarities at the amino acid level between these putative proteins are given in Table 5.2

Overall percentage identities between the amino acid sequences range from 20.6 to 25.7 while the percentage similarities range from 43.4-49.0.

Table 5.2; Percentage amino acid identity and similarity (italics) between AHV-1 peptide 1 (ORF 50) and the R transactivators from BHV-4, EBV, and HVS

Virus	AHV-1	BHV-4	EBV	HVS
AHV-1	-	***43.7***	***44.7***	***45.3***
BHV-4	21.6	-		***49.0***
EBV	20.6	21.4	-	***43.4***
HVS	22.9	25.7	22.7	-

A similar analysis of ORF A7 (peptide 5) revealed DNA homology to EBV ORF BZRF2 and to the family of cellular C-lectin proteins (Spriggs et al, 1996). A multiple alignment of the amino acid sequences with gaps inserted to optomise the alignment of peptide 5(AHV-1), EBV ORF BZRF2 and the human CD23 protein are shown in figure 5.6. The overall amino acid identity/similarity between the various ORFs is similar to that between the R transactivators described above, however in the case of the lectin homologs, there are also conserved structural motifs in the extracellular domain of the protein.

Published C-type lectin sequences contain a highly conserved WIGL motif indicated in bold type in figure 5.6. In the EBV this motif has been altered to WVGV, whereas

in AHV-1 the sequence is WIGT. Furthermore there are six cysteine residues conserved between the lectins which are also highlighted in bold in figure 5.6.

Figure 5.6; Alignment of the AHV-1 peptide 5 (ORF A7) with the EBV ORF BZLF2 and the C-type lectin protein, human CD23.

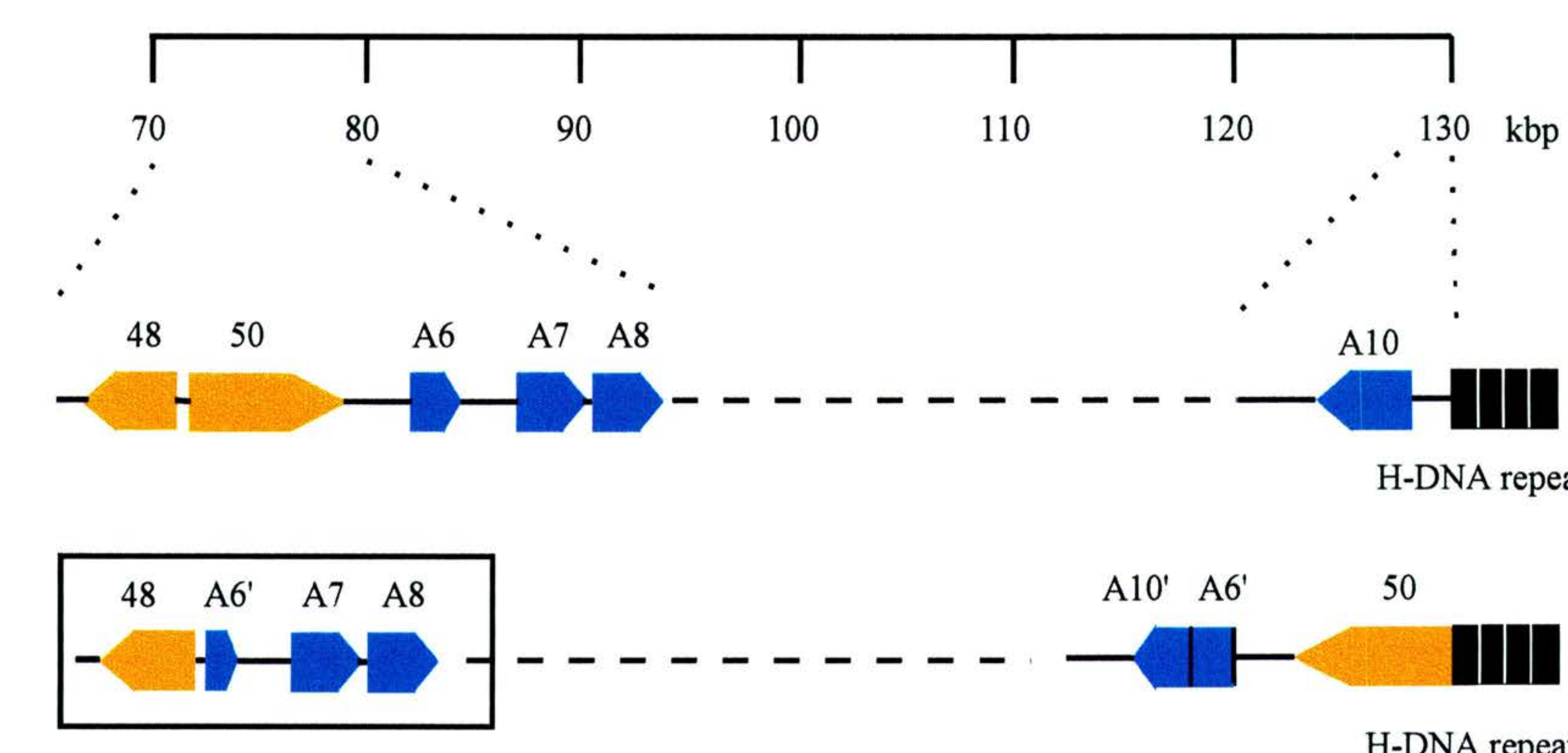
Conserved motifs are in bold. + indicates that an amino acid in this position is shared by two of the proteins while * indicates that it is present in all three.

```
AHV1    MLLPRKALLVDIFFILAATNLMIAAFALGCLAFYKQLVYITIGNLTFPHQSGDEVIRAMY
EBV     MV-SFKQVRVPLFTAIALVIVLLLAYFLPP--RVRGGGRVAAAAITWVPKPNVEVWPVDP
CD23    ------------------------------------------------------------
         +    +   +  +   +       +  +                 +       ++

AHV1    IPPVN----DSVDF-NPGFRLS-WLNTLSPLSDGPYDSWSQCEICPGR---FVGQKACYY
EBV     PPPVNFNKTAEQEYGDKEVKLPHWTPTLHTFQVPQNYTKANCTYCNTREYTFSYKGCCFY
CD23    ----------------------------------------VCNTCPEKWINFGRK--CYY
         ++++               +  +  ++             *  *+ +   *  +  *+*

AHV1    VPPKTYSFQNCFFACKNISKCFYLYTPQNITDPFFDHTLRD-QDIWIGTFFKKL---NAA
EBV     FTKKKHTWNGCFQACAELYPCTYFYGPTPDILPVVTRNLNAIESLWVGVY--RV---GEG
CD23    FGKGTYQWVHARYACDDM-EGQLVSIHSPEEQDFLTKHASHTGS-WIGL--RNLDLKGEF
        + ++++ +  +  **     + + + + +   ++ +       + *+*     +   ++

AHV1    LSTIDNNFDYT-------AWDELSVYCAYLTRRSRSTVYFTDCTT-SKLCLCGQEDFTPA
EBV     NWTSLDGGTFK-------VYQIFGSHCTYVSKFSTVPVSHHECSF-LKPCLCVSQRSNS-
CD23    IWVDGSHVDYSNWAPGEPTSRSQGEDCVMMR-GSGRWN-DAFCDRKLGAWVCDR------
         ++     ++             +  * +    *   +    *   +  ++*
```

5.3.4 PCR using primers V0144 and Y0332 (see appendix 6.1)

The results are presented in figure 5.3. PCR using primers V0144 and Y0332 only amplified DNA obtained from the wild-type isolates and cell associated (CA) C500 but not from the intermediate or the high passage cell free (CFA) C500. This indicated that these sequences are not adjacent in the intermediate and high passage C500 genome and that the block of DNA which is translocated is not also duplicated in its original position.

Figure 5.5. Diagrammatic representation of the genomic rearrangemnets described here.



5.3.5 Genome organisation at the left end of L-DNA

PCR using primers Lucrr/Lucs4 amplified a 170bp fragment with sequences homologous to the predicted AHV-1 sequences. However the LUCS2/LUCRR primers amplified a 250bp fragment in the intermediate passage cell free C500. The PCR fragment from low passage C500 and wild-type isolates was 450bp. The sequence of the 250bp PCR product showed that this fragment was made up of sequences from position 609-676 of the H-DNA and 72356-72565 of L-DNA. The orientation of the sequences in the H DNA fragment showed that this sequence was derived from the last terminal repeat at the left end of the genome. The L-DNA sequences were therefore translocated to the left end of the genome. These results indicated that LUCS2 had annealed at the correct position in the L-DNA and that the same primer inappropriately annealed within the terminal repeat. The size of the fragment translocated from the middle of the genome could not be established from this result. PCR was therefore conducted using intermediate passage DNA and primers designed from position 72450 (reverse) in conjuction with primers, 1040R, 1991R and 3153R to establish the size of sequences translocated to the left end of the genome in the cell free virus (see appendix 6.1). It was assumed that sequences translocated to the left end of the genome would be reversed in orientation as is shown to occur in the attenuated virus. A control reaction was also conducted with the three primers and RP2 as the upstream primer (section 5.2.2.8). The results showed that RP2 amplified fragments of predicted sizes with the 3 primers. Primer 72450 only amplified a fragment, of approximately 1350bp with the 1040 reverse primer. This fragment was cloned into pGEM-T and sequenced. The sequences

generated showed that the 72450 primer had annealed at the correct position in the middle of the genome but had also inappropriately annealed down stream of its correct position. The fragment did not contain sequences from the either end of L-DNA.

5.3.6 Sequence homologies for wild-type isolates

The results of ORF50 and A7 PCR are presented in figures 5.1 and 5.7 respectively. The results show PCR amplified fragments of the predicted sizes. The sequences generated from these fragments were homologous to the published sequences. There was therefore no significant difference between viral DNA obtained from the wild-type isolates and the virulent C500 sequences described by Ensser et al, 1997 in respect to the ORF 50 and ORF A7.

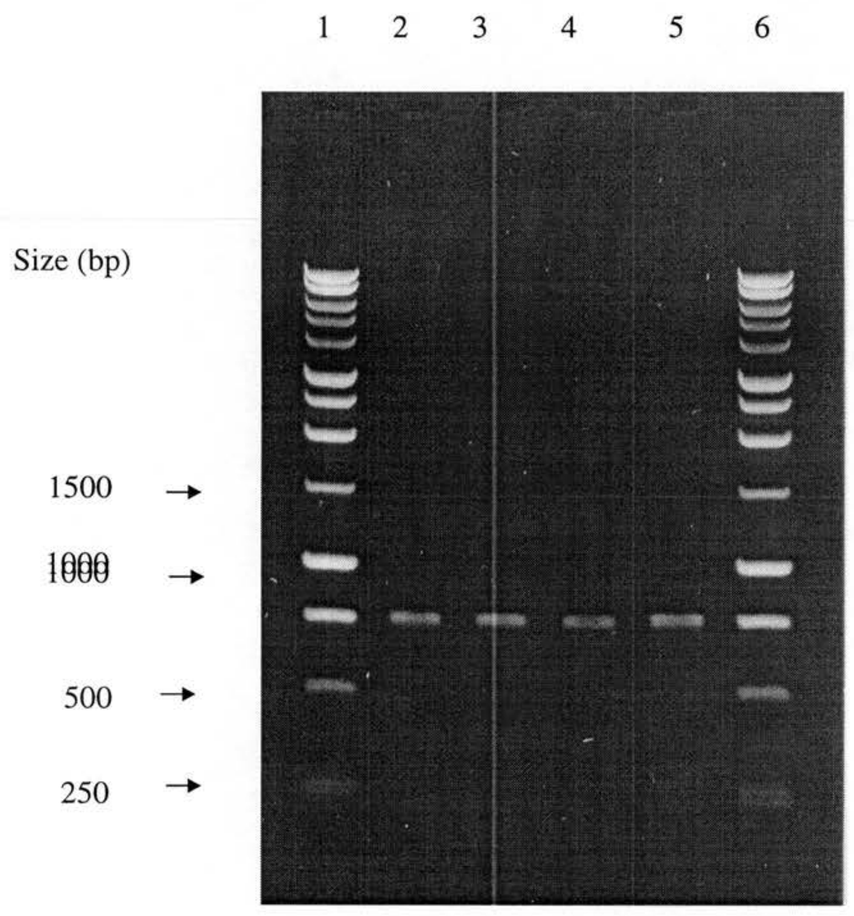
5.3.7 PCR to amplify ORF A8 (gp350/220

Using primers 14 and 14R a fragment of expected size was amplified using DNA from the wild-type virus and the low passage C500. In the intermediate passage and high passage cell free C500, two fragments of different sizes were observed; one fragment was equivalent in size to the fragment from the wild-type and low passage virus and the other fragment was smaller (approximately 1350bp). When WC11 DNA was amplified using the same primers, a PCR product equivalent in size to the smaller PCR fragment observed in high passage C500 was amplified (figure 5.8). One possibility was that this WC11 fragment had suffered a deletion compared with

the virulent virus or the PCR amplified a different fragment. The fragments generated from wild-type viral DNA and WC11 were extracted from gels, Genecleaned and precipitated with ethanol to remove residual salts (Chapter 2). The DNA concentration was adjusted to 200 ng μl^{-1}. Restriction maps of AHV-1 L-DNA sequences between 77431-78989bp showed that this region contained 2 restriction sites for Sma I and Stu I and one restriction site for Xba I Dra I, Kpn I and Pst I. 200ng of DNA extracted from the wild-type, low passage cell associated C500 and WC11 were digested with these enzymes in appropriate buffers to generate restriction maps to determine homology to C500 described by Ensser et al, (1997).

The restriction map profiles for the wild-type viral DNA were homologous to the low (CA) passage C500 as the restriction fragment sizes were as predicted from the database. However, the pattern for WC11 was different. The WC11 fragment was therefore cloned into pGEM-T and sequenced. The sequence was compared with the known AHV-1 L-DNA sequences in the database and the results indicated that primer 14 had annealed inappropriately at position 21694-21713 and position 22976-22996. The sequence of the PCR product amplified using primers 14F/R was identical to the AHV-1 DNA polymerase sequence. These sequences were therefore not related to the A8 sequence. It is not clear why the WC11 DNA was PCR negative for gene A8. It is possible that the sequences are deleted in this attenuated virus. It is likely that the smaller fragment in the PCR using 14/14R with CA DNA is also part of the polymerase gene.

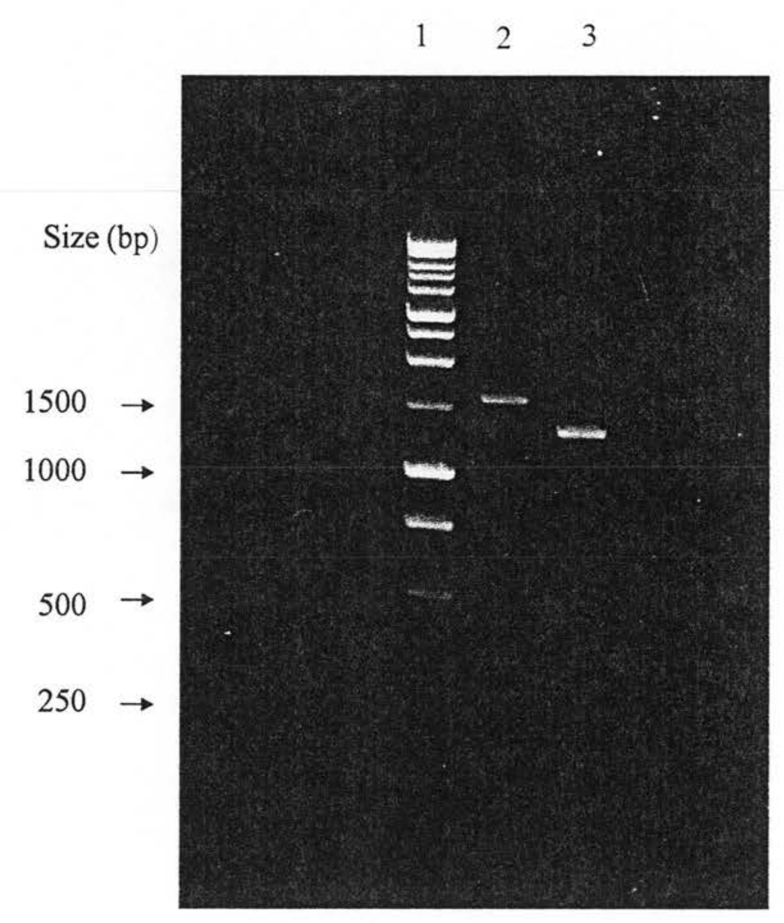
Figure 5.7 ; PCR of the four wild-type isolates using ORF A7 primers



Lane1&6- Molecular wt. markers

Lane 2-5-PCR results on all the four isolates

Figure 5.8. PCR on viral DNA using primers for ORF A8.



Lane 1= Molecular Wt. Markers

Lane 2= CA viral DNA

Lane 3= WC11 viral DNA

5.4 DISCUSSION

The publication of the complete sequence of the AHV-1 genome (Ensser et al, 1997) raised questions about the validity of the clones described by Handley et al, (1995). While clone vir 1 corresponded to the Hind III fragment at 73564-77088bp, the clones vir2 and att 1 were shown to be admixtures of sequence from this Hind III fragment and sequences from the 3'end of the genome. The question as to whether these clones were chimaeric artefacts of cloning or were genuine clones from a rearranged viral genome was addressed here by sequencing PCR fragments obtained with pairs of primers which should not amplify DNA from virulent virus according to the sequence described by Ensser et al, (1997).

The results show that rearrangements take place in the C500 viral genome during serial passage in tissue culture. The DNA sequence rearrangement results in AHV-1 virus which is no longer pathogenic for rabbits (Handley et al, 1995). The authors suggested that the rearrangements occur at the 5'of the genome and that the rearrangements involve deletions and truncations of the ORFs designated peptide 1 and peptide 5. The genomes of γ-herpesviruses are generally collinear. These peptides were therefore considered on positional and structural grounds to be equivalents of the HVS transforming proteins STP (saimiri transforming proteins STP-A11 and STP-C488, Murphy et al, 1989, Biesinger et al, 1990) and TIP (Tyrosine Kinase interacting protein, Biesinger et al, (1995). The genes for these proteins are located close to the 5'end of the HVS L DNA (Albrecht et al, 1992)

However, the sequences of the PCR fragments presented here, together with the ladder of bands produced on hybridisation blots of viral DNA which has been digested with restriction enzymes such as Hind III, Bam H1 and Eco R1 which do not

cut within the tandem repeats, with peptide 1 DNA (Handley et al, 1995), demonstrates the proximity of P-1 to terminal repeats in the attenuated virus. The presence of a product when primers Y0332 and V0144 were used to amplify CA and wild-type viral DNA and its absence in the CFA DNA would indicate a translocation rather than a duplication/ translocation.

The sequence from the repeat unit starts at -152bp with respect to the initiation codon of peptide 1 (ORF 50) (figure 5.2). This sequence may not be long enough to encode crucial upstream regulatory sequences. There is therefore need to determine whether the regulatory factors, i.e. promoter for this immediate early protein are functional. This is discussed in chapter 6.

Whatever the consequence of this rearrangement may be for AHV-1 pathogenicity, the attenuated viruses are clearly able to productively replicate in vitro producing viable virions that cause cytopathology in cell cultures.

The sequence of the PCR product obtained using LUCS2/LUCR with intermediate viral DNA provided evidence that the block of DNA at around 75,000bp in the genome may also be translocated to the left end of the genome as indicated by Handley et al, (1995). However, no further PCR products using primer 1991R, 1090R , 2053R and primer RP2 were obtained which could define the extent of the translocation to the left end.

Protein alignment for peptide 5 show a high percentage of conserved amino acid motifs with BZLF2 amino acid sequences in EBV and the human CD23 , indicating that this peptide in AHV-1 may play the same functional role as this protein.

There are no sequence differences in DNA derived from wild-type isolates and the laboratory adapted virulent C500 isolate in respect of ORFs, 50 (peptide 1), A7

(peptide 5) and A8 that have been shown to be truncated or deleted in the attenuated virus.

It is now clear however that P-1 is the AHV-1 equivalent of the R transactivators of EBV, BHV-4 and HVS while peptide 5 is a homologue of C-type lectins. The genes for these proteins are located in the central position in the unique DNA of both EBV (Baer et al, 1982) and HVS (Albrecht et al, 1992). Furthermore, Ensser et al (1997) have now sequenced the whole genome from the virulent albeit cell free C500 isolate and place the genes for the peptides 1 and 5 at 72825-74903bp and 76569-77300bp respectively, the total length of the unique DNA being 130kbp.

AHV-1 in wildebeest calves multiplies most probably in the upper respiratory tract (Mushi et al, 1980b). The virus then infects peripheral blood lymphocytes and a vireamia can be detected in such animals (Plowright et al, 1960, Plowright et al, 1965). Infected wildebeest continue to shed cell free virus in nasal and ocular discharges for a limited period of time (Mushi et al, 1980b). Cessation in virus shedding is associated with development of humoral and surface antibody (Plowright 1967, Rurangirwa et al, 1982).

AHV-1 infection in cattle leads in development of severe MCF and recovery is rare (Plowright 1986). Cattle infected with AHV-1 do not shed cell free virus (Mushi et al, 1981c), suggesting that lytic replication does not occur in infected cattle.

One cow that recovered from AHV-1 was shown to transmit disease to 3 out of four offspring over a period of 4 years though the animal had a high level of humoral neutralising antibody to AHV-1 indicating that cell mediated immunity may be important in the maintenance of latency in cattle that recover from clinical MCF (Plowright et al, 1972).

Since R has been shown to be crucial in lytic phase of infection in EBV this protein may be important in the epidemiology and pathogenesis of MCF.

The results presented in Chapter 3 show that peptide 1(R transactivator) is expressed *in vitro* (i.e. in infected cell culture) but not *in vivo* (in peripheral blood lymphocytes derived from cattle infected with virulent AHV-1) though failure to detect proteins may be due to very low frequency of virus infected cells.

C-type lectins are transmembrane glycoproteins, common in eukaryotic cells (Yokoyama et al, 1989), and also in blood cells that possess natural killer characteristics (Spriggs et al, 1996). The glycoproteins present on these cells are important in MHC class II antigen recognition and lysis (Li et al, 1997).

In cattle the target cell for AHV-1 is the T lymphocyte (Rurangirwa et al, 1981, Mushi et al, 1984) although probably limited replication occurs in the oculo-nasal epithelia. Expression of this protein on the surface of infected T cell may therefore phenotypically alter such cells so that they acquire NK function.

The mechanisms involved in the pathogenesis of AHV-1 are poorly understood as direct viral cytopathology cannot be demonstrated. The most probable hypothesis is that infected T cells are deregulated so that they acquire NK activity (Reid and Buxton, 1984). It is therefore tempting to speculate that infection of the target cell leads to expression of C-type lectins and that such altered T cells are responsible for the cell destruction observed in MCF.

Ensser et al (1997) also showed that AHV-1 encodes a putative glycoprotein with limited homology to the gp350/220 described in EBV. The results presented here show that this sequence encoding this putative glycoprotein was amplified in virulent viral DNA but not in DNA derived from the attenuated virus. This result suggests that this protein may be truncated or deleted during the process the attenuation.

In EBV this glycoprotein is essential in the attachment of the virus to B cells during the infection cycle *in vitro*. This glycoprotein may have the same function in AHV-1 but may only be important *in vivo* as lytic virus replication occurs following infection of cell cultures with the attenuated virus (Plowright et al, 1965a,b).

Another ORF was shown to share positional but limited structural homology to the ORF 50 in HVS and BRLF1 in EBV. The ORF was designated ORF50 to denote its positional albeit limited structural similarity to the HVS ORF. In HVS, ORF50 codes for a transactivator that is essential in the transition from latency to lytic replication in B cells infected *in vitro*. This putative protein is discussed in chapter 6.

CHAPTER 6

ACTIVITY OF THE PUTATIVE ORF 50 PROMOTER

6.1 INTRODUCTION

Malignant catarrhal fever is a pathological syndrome characterised by generalised lymphocytic hyperplasia and disseminated necrosis (Plowright 1986). Virus cannot be demonstrated in tissues of infected cattle and viral antigen can only be demonstrated in 1 out of 10^4 lymphocytes from infected rabbits (Edington and Patel, 1980) and viral DNA in the same proportion of cells (Bridgen et al, 1989). The pathogenesis of this disease is therefore poorly understood and no direct virus cytopathology can be demonstrated. One hypothesis is that infection of T cells leads to deregulation of these cytotoxic cells such that they acquire natural killer cell characteristics and hence the cellular necrosis observed (Reid and Buxton, 1984). In wildebeest associated MCF however, the virus is readily isolated by co-cultivation of lymphocytes obtained from infected animals with cell cultures (Plowright et al 1960, 1965b). In the natural host, the wildebeest, animals acquire infection *in utero* or perinatally and such animals continue to shed cell free virus in nasal and ocular secretions for a few months (Plowright 1965a, Mushi et al, 1980, 1984). Cessation in virus shedding is accompanied by the development of humoral and mucosal surface antibody (Mushi et al, 1981c, Rurangirwa et al 1982, Plowright, 1965b). AHV-1 has however been isolated from adult wildebeest following treatment with corticosteroids or under the stress of confinement, malnutrition and pregnancy (Rweyemamu et al, 1974, Plowright, 1965b). Such animals may be latently infected and remain carriers although virus shedding can be induced by stress.

OHV-2 has been shown to immortalise lymphocytes derived from MCF affected rabbits (Reid et al, 1983). Similar cells are immortalised by AHV-1 (Reid-personal

communication). The LCL are latently infected as viral DNA can be detected in these cells and such cells induce typical MCF following infection of rabbits (Bridgen et al, 1989, Reid et al, 1983, 1989). Intact virus cannot be detected as viral DNA is probably present in an episomal form (Reid-personal communication)

In EBV, a gene product encoded by ORF BZLF1 has been shown to induce the transition from latency to the lytic productive cycle in latently infected lymphocytes (Chevalier-Greco et al, 1986, Countryman et al, 1987). Another transacting immediate early protein, designated R and transcribed from the EBV BRLF1 gene (Hardwick et al, 1989) has been shown to induce productive cycle of EBV replication only in conjunction with the EB1 (Manet et al, 1989). Indeed, this transactivator has been shown to interact with the BZLF1 protein and retinoblastoma protein (Rb) in a mechanism that leads in the hyperphosphorylation of Rb and thence induction of the lytic cycle (Cannell et al, 1996). This transactivator may therefore be important in the pathogenesis of EBV.

AHV-1 has now been shown to contain putative homologues of these genes (Chapter 5). ORF 50 has been designated the R transactivator while ORF A6 may be the equivalent of BZLF2, the Z transactivator.

Sinclair et al (1991) have reported that the BZLF1 gene product in EBV can activate the BRLF1 promoter and have suggested that disruption of latency may be mediated at the BZLF1 promoter, with the Z (previously designated EB1) protein activating the BRLF1 promoter. The authors also showed that R is transcribed together with Z as a bicistronic mRNA from the BRLF1 promoter and that this promoter is activated by both viral proteins.

The results presented in Chapter 5 show that attenuation of AHV-1 is associated with

The results presented in Chapter 5 show that attenuation of AHV-1 is associated with translocation and inversion of ORF 50 together with a short stretch of sequence upstream of ORF 50 which is also inverted and inserted between ORF 50 and the terminal repeats at the 3' end of the genome.

Analysis of the AHV-1 genome suggests that ORF 50 may be transcribed as two exons (Ensser et al, 1997). Similarly, ORF 50 in HVS is transcribed from two distinct promoters to yield two gene products, ORF 50a and ORF 50b (Whitehouse et al, 1998). ORF 50a was shown to encode an IE protein, while ORF 50b, is a DE protein with a promoter within the ORF 50a. Transient transfection assays showed both promoters to be functional and that the ORF 50a gene product acts as an immediate early transactivator. The authors however did not establish the role of ORF 50b but suggested that this may act as a negative regulator of the ORF 50a promoter. Another gene product, encoded by ORF57 in HVS has been shown to repress the transactivation capability of ORF 50a via post-transcriptional splicing and slightly activate ORF 50b transcription (Whitehouse et al, 1998). This repression was also dependent on the presence of an intron within the ORF 50a.

Since the HVS and AHV-1 genomes are generally homologous, it was assumed that ORF 50a in AHV-1 could possess the same functional role as in HVS.

In HVS, 50a is transcribed from a promoter located at least 417bp upstream of the start codon within the adjacent ORF 49 (Whitehouse et al, 1998). ORF 50b is a delayed early spliced transcript with a promoter located within the non-coding 50b ORF. The function of this latter protein has not yet been established.

Sinclair et al, (1991) reported that BZLF1 can reactivate BRLF1 *in vitro*. Zalani et al, (1992) showed that BRLF1 promoter contains two transactivator binding sites and

suggested that R can activate its own promoter. R, transcribed by ORF 50a in HVS has a classic eukaryotic promoter motif, putative TATA and CCAAT consensus sequences at -99bp and -183bp upstream of the ATG translation codon respectively.

In EBV infection of lymphocytes *in vitro* is characterised by immortalisation and virus latency whereas infection of the epithelial cells *in vitro* is associated with productive infection and cell lysis. It has now been shown that the R transactivator can induce the lytic cycle genes and disrupt latency in B cells as well (Ragoczy et al, 1998).

It is therefore possible to speculate that the latency observed in AHV-1 could be due to the inactivity of the promoter controlling ORF 50.

There is no information on the functions of the ORF 50 promoter or gene product in AHV- 1. The results presented in chapter 3 indicate that this protein is transcribed and translated *in vitro*. Since it is now clear that this protein is an I.E. protein it would be useful to determine whether this protein is transcribed in cultures infected with the virulent and the attenuated viruses in the presence of cycloheximide. Cycloheximide inhibits translation of mRNA. There is therefore increased production of viral mRNA in cultures infected with virus in the presence of cycloheximide. An aim of this chapter is therefore to determine whether the R transactivator is transcribed in cultures infected with the virulent and the attenuated derivatives of AHV-1 in the presence of cycloheximide.

The other aim of this chapter is to determine whether the putative ORF 50 promoter in the virulent virus and the truncated promoter in the attenuated virus are functional using the American firefly (*Photinus pyralis)* luciferase gene as a reporter gene in transient transfection assays.

6.2 Materials and methods

6.2.1 Preparation of Immediate Early (I.E.) RNA.

Growth media (Iscoves modification of Dulbecco's medium (GIBCO), supplemented with 10% Foetal bovine serum, 100 IU/ml penicillin, 50 IU/ml streptomycin, 2 IU/ml fungizone (Rousell) containing cycloheximide at a concentration of 100 μg/ml was added to confluent monolayers of BT cells in 25cm^2 tissue culture flasks and infected at high multiplicity of infection (approximately 2 $TCID_{50}$/cell) with either WC11, C500 CA or CFA. After 8 hr, the medium was poured off and total cellular RNA prepared as described in 3.2.13 except that 10 ml of GITC was added on to the cell culture monolayers. The RNA precipitate was dissolved in 100μl of water and the RNA concentration obtained by spectrophotometry.

6.2.2 Analysis of RNA by Northern Blotting

10 μg of total RNA were run on a 1% denaturing gel as described in section 3.2.14. DNA was transferred to Hybond-N membrane (Amersham) by capillary blotting as described in section 3.2.16. DNA was labelled with [α^{32}P]dCTP using Rediprime (Amersham) and hybridised in RapidHyb hybridisation buffer (Amersham) to the transferred RNA at a temperature of 65^0 C (sections 6.2.5 and 6.2.6). The membrane was washed in 0.2x SSC, 0.1% SDS at 65^0 C and exposed to X-ray film overnight.

6.2.3 Isolation of AHV-1-derived promoter sequences

AHV-1 genomic DNA from virulent and attenuated virus was extracted as described previously (Chapter 3). The complete sequence for AHV-1 is available from Genbank (Ensser et al, 1997). The AHV-1 sequences upstream of the initiation codon of P-1, the R transactivator, were amplified from the CA and CFA viruses using *Taq* PCR (section 2.5.15.1). The primers used and the positions of these primers with respect to the AHV-1 L-DNA are listed below (see also appendix 6.1).

The primers were engineered to contain suitable restriction sites for ligation into the vector pGL2 which carries the luciferase gene.

6.2.4 Description of primers

The primers were designed to allow ligation of the PCR fragments into the plasmid pGL2 upstream of the luciferase reporter gene. The positions of the primers in the AHV-1 genome are shown diagrammatically in Appendix 6.1.

Lucs4 : This was used to prime the truncated part of the promoter of the R transactivator in attenuated AHV-1 (CFA).

```
5' (72345)- CATTCTGTAAACATACTCTCAATGTTTT-  (72372) 3'
          1 CATTCTGGTACCATACTCTCAATGTTTT 28
                  KpnI
```

Lucs3 : This was used to prime the promoter of the R transactivator of virulent C500 AHV-1 (CA).

```
5′ (71828) -AAGGGAGGTACCTGCTGCTATGGGAAAC - (71855) 3′
         1  AAGGGAGGTACCTGCTGCTATGGGAAAC 28
                  KpnI
```

Lucr2 : This is a primer reversed at start of exon 1 of the R transactivator.

```
5′  (72808) -AAATTTGACTACCCAAAATGAGTGCCAAC-  (72836) 3′
         29 AAATTTGACTACCCAACTCGAGTGCCAAC 1
                             Xho1
```

Lucr1 : A 5' primer for the promoter region of the R transactivator of AHV-1 for ligation into the luciferase reporter gene

```
5′  (72808) -AAATTTGACTACCCAAAATGAGTGCCAAC-  (72836) 3′
         29 AAATTTGACTACCCAAAGATCTGCCAAC 1
                              Bgl II
```

RP1 : This is a primer located in the terminal repeat H-DNA at a position close to the Sac I site as shown.

```
          RP1              Sac I
  TCTGTGAGGCTGAGCACCCG.........GAGCTC
-44                              -23
```

6.2.5 PCR

Taq PCR was used to amplify the viral sequences. Reactions were set up using primer pairs lucs4/lucr1 and lucs3/lucr1 to amplify fragments of 467 bp and 984 bp upstream of the initiation codon of the R transactivator (ORF 50 or P-1) in C500 (CA). These are coded pGL2-CA1 and pGL2-CA2 respectively in table 6.1.

Lucs4/lucr1 were also used to amplify the equivalent region in WC11 and the construct is coded pGL2-WC11. The region upstream of the R transactivator in its inverted orientation adjacent to the H-DNA in the attenuated C500 (CFA) genome were amplified using primer pair RP1/ lucr2 and is coded CFA in table 6.1.

6.2.6 Plasmids used for cloning ORF 50 promoter

The pGL2 Luciferase Reporter Vectors used were purchased from Promega. These plasmids are designed to support convenient analysis of promoter sequences using the firefly *Photinus pyralis* as the genetic reporter. Two plasmids were used as positive and negative controls in the experiment in order to assess the efficiency of the luciferase assay procedure. The genotype and cloning sites for the pGL2 plasmid are described in tables 2.2 and 2.3. Controls were:

pGL2 Control. This plasmid contains the enhancer and promoter sequences for the luciferase gene. Cells transfected with this plasmid will therefore produce luciferase enzyme.

pGL2 Basic. This plasmid does not contain either the promoter or the enhancer sequences. Cells transfected with this plasmid do not therefore produce luciferase.

6.2.7 Cloning of R transactivator sequences into pGL2 plasmids

The PCR fragments were digested with appropriate restriction enzymes and ligated into plasmids that had been linearised following digestion with the respective enzymes. Ligations were conducted with T4 ligase and the ligation mixture used to

the cells to grow in LB agar containing ampicillin. Single colonies were then grown in LB and the plasmid extracted as described previously. Colonies containing recombinant plasmid were selected following demonstration of insert in the recombinants after digestion with the appropriate restriction enzymes. Since two different restriction enzymes were used to make recombinant plasmids the ligation was assumed to be possible in only one direction. Large scale plasmid DNA was prepared and an aliquot sequenced using forward and reverse pGL2 primers to confirm presence and orientation of the inserts.

PCR fragments pGL2-CA1, pGL2-CA2 and pGL2-WC11 were digested with Bgl II and Kpn I before ligation into the pGL2 plasmid. Fragment pGL2-CFA was digested with SacI and Xho I.

6.2.8 Preparation of ^{32}P labeled probes for colony lifts and hybridisation

This method was used to confirm that competent JM109 cells transformed with pGL2 plasmids ligated with ORF 50 promoter sequences contained the desired insert. This technique was used since colonies transformed with recombinant plasmid could not be selected using the IPTG/X-gal "blue" or "white" marker system. DNA used as the probe were PCR products used to construct the respective recombinant plasmids. 10ng of probe DNA was denatured by boiling for 5 min in a total volume of 45 μl of water and then chilled quickly on ice. Denatured DNA was added to a Rediprime tube containing random hexanucleotides, dNTPs and polymerase enzyme provided in the "Rediprime DNA labelling kit" (Amersham). 5μl of α-P^{32}-dCTP at 3000Ci/mmol was added and the mixture incubated at 37^0C for 1 hour.

3000Ci/mmol was added and the mixture incubated at 37^0C for 1 hour.

6.2.9 Hybridisation of ^{32}P labeled probes

Hybond N membranes (Amersham) were placed on the bacterial colonies until the membranes were wet through. The orientation of the colonies on the plate and on the membrane was fixed by puncturing holes through the membrane. The membrane was then carefully peeled off the bacterial cells and placed on a Whatman 3MM filter paper soaked in 10% SDS for 3 minutes and then transferred to one soaked in 0.5 M NaOH/1.5 M NaCl for 5 minutes to lyse the colonies. The membrane was neutralised by being placed on a filter of 0.5 M Tris/HCl pH 7.0/1.5M NaCl for 5 minutes. It was then placed on a filter soaked in 2x SSC for 5 minutes before being allowed to dry in air. The membrane was wetted in 2x SSC and the bacterial debris wiped off carefully with tissues. The membrane was dried and the plasmid DNA fixed to the Hybond-N by exposure to UV for 1 minute on the surface of the transilluminator. The membranes were wetted by placing them briefly in 2x SSC and then prehybridised in RapidHyb^R (Amersham) (20 ml 100cm^{-2} of membrane) for 1hr at 65^0 C. The solution was decanted and fresh hybridisation buffer containing denatured probe added. Hybridisation was carried out for 2-16 hr at 65^0C and the filters washed twice in 0.1x SSC, 0.1% SDS at the same temperature for 30 min. The filters were dried with paper towels and bound radiolabeled probe detected by exposing the membrane to X-ray film in the dark for 12 hours. The films were developed using an automatic processor (Compact X2, Kodak) and the colonies containing insert determined by aligning the bacterial plates with the autoradiograph.

6.2.10 Transfection of BT cell cultures with plasmid

Five micrograms of plasmid DNA were added to 150μl serum free MEM. Three hundred microlitres of Superfect Transfection Reagent™ (Qiagen) was added to the DNA and the contents vortexed lightly. The mixture was left to stand at room temperature for 10 minutes and 1 ml of ISCOVES media (GIBCO) added. Media was removed from BT cell culture monolayers showing approximately 80% confluence in 60mm culture flasks and the cells washed once with PBS. The monolayer was overlaid with the plasmid DNA/Transfect mixture and the cultures incubated for 3 hours at 37^0C. DNA/Transfect mixture was removed from the culture flasks and 10ml of media containing antibiotics and 10% foetal calf serum added. The cultures were incubated for 48 hours at 37^0C when the cells were harvested for luciferase enzyme activity assay.

6.2.11 Transfection and infection / Infection and transfection

This experiment was carried out to determine whether virus replication would influence the ORF 50 promoter activity *in vitro*. BT cultures were infected at a multiplicity of infection of approximately $2TCID_{50}$/cell with either WC11 or C500 (CA) and the cells transfected 24 hr later. The cells were assayed for luciferase activity 24 hr after transfection. For the transfection and infection assay, the cells were transfected with the constructs and then infected 24 hr later at a multiplicity of infection of approximately $2TCID_{50}$/ cell with either the virulent C500 or attenuated WC11 isolate. The cells were harvested for assay after 48 hr.

6.2.12 Luciferase activity assay.

This assay was performed using reagents purchased from Promega. The method used was as described by the manufacturers. Media was removed from cell culture monolayers and the cell sheet rinsed twice with PBS, taking care not to dislodge the cells. Two hundred and fifty microlitres of 1x cell culture lysis reagent was added to a 60mm cell culture flask and the attached cells scraped from the culture flasks. The cells and the solution were spun for 5 seconds in a microfuge at 12,000 g to pellet the cell debris and the supernatant transferred to a new tube. This cell extract could be used immediately or aliquoted and stored at -70^{0}C until required. For assay, 20μl of cell extract was mixed with 100μl of luciferase assay reagent at room temperature. The sample was then assayed in a liquid scintillation counter (Packard, Model 2500 TR) set up with the coincidence circuitry switched off. The counts were recorded every 30 sec for 4 minutes. Assays were conducted in replicate and cells not transfected with plasmid (mock transfected) were included as controls in all cases.

6.3 RESULTS

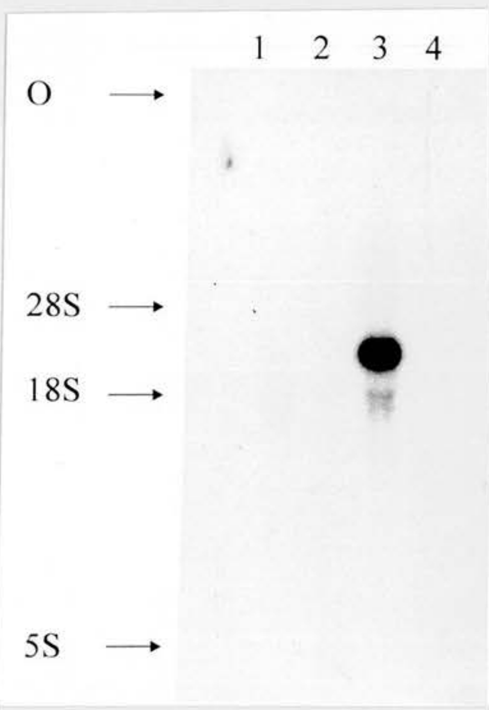
6.3.1 Northern analysis of RNA from infected BT cells.

The results of Northern analysis of RNA from cell cultures infected with WC11, C500 (CA) or C500 (CFA) in the presence of cycloheximide are presented in figure 6.1. Substantial amounts of RNA which hybridised to an AHV-1 R transactivator gene probe were produced in only in those BT cells infected with the virulent, low passage form of AHV-1 C500 (CA). The attenuated forms, WC11 and C500 (CFA) were negative, as were the uninfected cells.

Figure 6.1. Northern blot of total RNA from cycloheximide-treated BT cells.

BT cells were treated with virus as indicated below and in the text. The blot was hybridised with a ^{32}P labelled DNA probe from ORF 50 (peptide 1). The blot was washed in 0.2 x SSC, 0.1% SDS at 65^0C and exposed to X-Ray film overnight at –70^0C. Arrows indicate the positions of the ribosomal RNAs. The 28S RNA is approximately equivalent to a 5kbp dsDNA fragment while the 18S is equivalent to a 2 kbp ds DNA fragment.

Track no.	Sample	Track no.	Sample
1	Control BT	3	BT + C500 (CA)
2	BT + WC11	4	BT + C500 (CFA)



6.3.2 PCR of ORF 50 promoter fragments

PCR fragments of the predicted sizes were amplified using different combination of primers and target DNA (table 6.1). The amplified products were used for ligation into the pGL2-basic plasmid.

Table 6.1. Fragments used for ligation into the pGL2

Target DNA	Primers	Size (bp)	Position in genome	Code
C500 (CA)	LucS4/LucR1	467	72357-72824	pGL2-CA1
C500 (CA)	LucS2/ LucR1	984	71851-72824	pGL2-CA2
C500 (CFA)	RP1/Lucr2	344	1150-1035* + 72550-72824	pGL2-CFA
WC11	LucS4/LucR1	467	72357-72824	pGL2-WC11

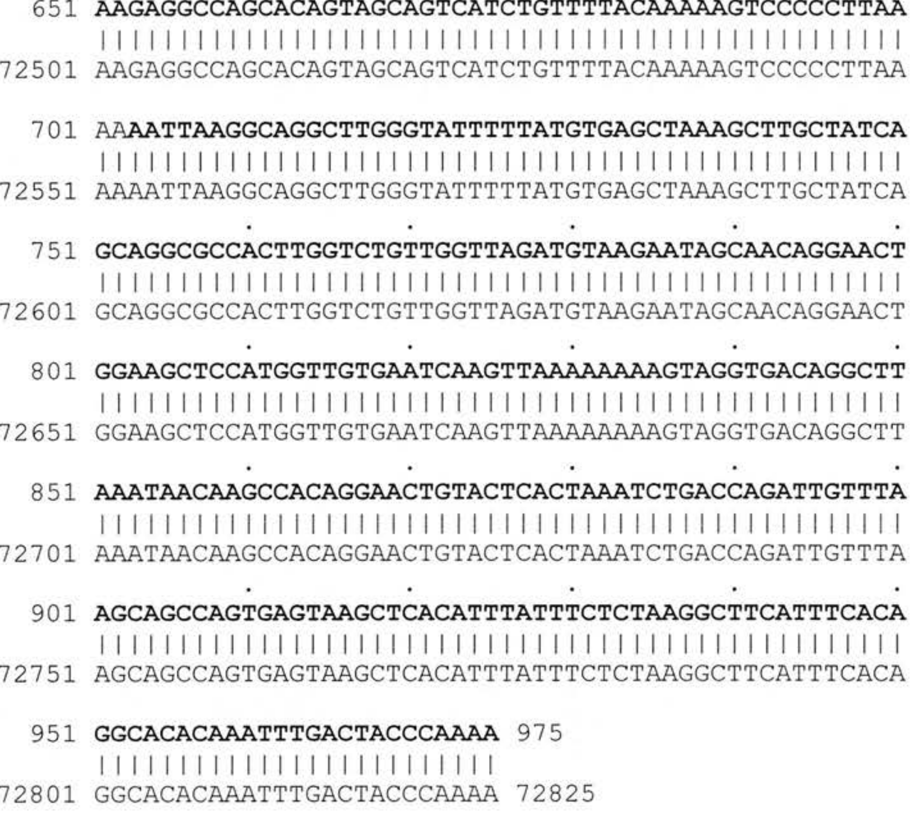
*position in H-DNA. CA = Cell associated. CFA = Cell free attenuated.

6.3.3 Sequences of ORF 50 promoter fragments cloned into PGL2

The pGL2 clones were sequenced to confirm the identity of the inserts and the sequences are given in figure 6.2. The sequences of the L-DNA were identical to those of Ensser et al. (1997) The results also show that the promoter for the R transactivator is truncated and inserted at the right end of the L-DNA adjacent to the terminal repeats (see also figure 5.2).

Figure 6.2a. Sequence homology of PCR fragments cloned into pGL2. The complete sequence of the insert in pGL2-CA2 is shown and the DNA homology to L-DNA of AHV-1 indicated. The sequences of clone pGL2-CA1 and pGL2-WC11 are represented in bold.

```
    1 GAAACTTAACTACATTTTTATTTCCACCCATAAAAGCTCCCCTCTCTTTA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
71851 GAAACTTAACTACATTTTTATTTCCACCCATAAAAGCTCCCCTCTCTTTA
               .         .         .         .         .
   51 ATCTTTTTTAGCAGCCTTACATCTAATCTACAGAGGTCCCTTTTAGACTC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
71901 ATCTTTTTTAGCAGCCTTACATCTAATCTACAGAGGTCCCTTTTAGACTC
               .         .         .         .         .
  101 TAGAGGTATTTTATAGGTCACAGCAAACTGCATCATGTCTTCAAAAATGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
71951 TAGAGGTATTTTATAGGTCACAGCAAACTGCATCATGTCTTCAAAAATGT
               .         .         .         .         .
  151 CCTCCAAAGCACCAGTTACTGTCTGCTGCATTTTGTAAAATAGCCAGCAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72001 CCTCCAAAGCACCAGTTACTGTCTGCTGCATTTTGTAAAATAGCCAGCAG
               .         .         .         .         .
  201 AAAGTTATACATATAGCTAATAAACTCGCTTCTTTTTTAGCTTCTATGCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72051 AAAGTTATACATATAGCTAATAAACTCGCTTCTTTTTTAGCTTCTATGCT
               .         .         .         .         .
  251 AAAGCCCCCTTCAAAGTGCGACCCCATGACTATGTCACGGAGCAGATCCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72101 AAAGCCCCCTTCAAAGTGCGACCCCATGACTATGTCACGGAGCAGATCCT
               .         .         .         .         .
  301 TAGAAACTGGAACACTGTTACACAAGACAGCACTGTTGTAAACTGAAGAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72151 TAGAAACTGGAACACTGTTACACAAGACAGCACTGTTGTAAACTGAAGAC
               .         .         .         .         .
  351 AATGTAAACTCATAAGAGGAAATTTCATGTGGGTTTAATCTAGGCATCCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72201 AATGTAAACTCATAAGAGGAAATTTCATGTGGGTTTAATCTAGGCATCCT
               .         .         .         .         .
  401 ATAGGAAATTTGTGCCTTCTGTAGCCCCTTCACTGTTTCCAAGCACTCCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72251 ATAGGAAATTTGTGCCTTCTGTAGCCCCTTCACTGTTTCCAAGCACTCCA
               .         .         .         .         .
  451 TACATCCACATTCACATTCCAACAGCAGAGCTAACCTCCTACTGCATTCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72301 TACATCCACATTCACATTCCAACAGCAGAGCTAACCTCCTACTGCATTCT
               .         .         .         .         .
  501 GTAAACATACTCTCAATGTTTTCTGGATACACCCGTTGAAAAATTTCATC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72351 GTAAACATACTCTCAATGTTTTCTGGATACACCCGTTGAAAAATTTCATC
               .         .         .         .         .
  551 AAAACAATATTCTGCTAAACTTCTTACCAGAATTGCCATCTGGATAACTC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72401 AAAACAATATTCTGCTAAACTTCTTACCAGAATTGCCATCTGGATAACTC
               .         .         .         .         .
  601 TGTCCTTTTCAATCATATTGTCTTCTGTTTGAATCAGGCTAACTAGCACC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72451 TGTCCTTTTCAATCATATTGTCTTCTGTTTGAATCAGGCTAACTAGCACC
```



```
  651 AAGAGGCCAGCACAGTAGCAGTCATCTGTTTTACAAAAAGTCCCCCTTAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72501 AAGAGGCCAGCACAGTAGCAGTCATCTGTTTTACAAAAAGTCCCCCTTAA

  701 AAAATTAAGGCAGGCTTGGGTATTTTTATGTGAGCTAAAGCTTGCTATCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72551 AAAATTAAGGCAGGCTTGGGTATTTTTATGTGAGCTAAAGCTTGCTATCA
                .         .         .         .         .
  751 GCAGGCGCCACTTGGTCTGTTGGTTAGATGTAAGAATAGCAACAGGAACT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72601 GCAGGCGCCACTTGGTCTGTTGGTTAGATGTAAGAATAGCAACAGGAACT
                .         .         .         .         .
  801 GGAAGCTCCATGGTTGTGAATCAAGTTAAAAAAAAGTAGGTGACAGGCTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72651 GGAAGCTCCATGGTTGTGAATCAAGTTAAAAAAAAGTAGGTGACAGGCTT
                .         .         .         .         .
  851 AAATAACAAGCCACAGGAACTGTACTCACTAAATCTGACCAGATTGTTTA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72701 AAATAACAAGCCACAGGAACTGTACTCACTAAATCTGACCAGATTGTTTA
                .         .         .         .         .
  901 AGCAGCCAGTGAGTAAGCTCACATTTATTTCTCTAAGGCTTCATTTCACA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72751 AGCAGCCAGTGAGTAAGCTCACATTTATTTCTCTAAGGCTTCATTTCACA

  951 GGCACACAAATTTGACTACCCAAAA 975
      |||||||||||||||||||||||||
72801 GGCACACAAATTTGACTACCCAAAA 72825
```

Figure 6.2b. Sequence of the insert in clone pGL2-CFA showing the homology of the first 77 bp to AHV-1 H-DNA (in bold typeface) and the homology of the rest of the clone to AHV-1 L-DNA. The start codon for the R transactivator is underlined.

```
    1 ......TTCGGGCCAGAGA.CCGGAGAGAGGGGAAAAAAAACCAGGGG
            || || ||||||| ||||||||||||||||||||||||||||
 1113 GAGCTCTGTTGGGCCCAGAGACCCGGAGAGAGGGGAAAAAAAACCAGGGG
                .         .         .         .         .
   42 GACGGGCCGCGGGGGGCTCGGGGGCCGGGCGATGCC..............77
      ||||||||||||||||||||||||| ||||     |||
 1063 GACGGGCCGCGGGGGGCTCGGGGGGCGGGGAGGGCCGCGGAGCGACAAAA

   78 .......................AAGTTAAAAAAAAGTAGGTGACAGGCT
                             ||||||||||||||||||||||||||
72650 TGGAAGCTCCATGGTTGTGAATCAAGTTAAAAAAAAGTAGGTGACAGGCT
                .         .         .         .         .
  105 TAAATAACAAGCCACAGGAACTGTACTCACTAAATCTGACCAGATTGTTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72700 TAAATAACAAGCCACAGGAACTGTACTCACTAAATCTGACCAGATTGTTT
                .         .         .         .         .
  155 AAGCAGCCAGTGAGTAAGCTCACATTTATTTCTCTAAGGCTTCATTTCAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
72750 AAGCAGCCAGTGAGTAAGCTCACATTTATTTCTCTAAGGCTTCATTTCAC
                .         .         .         .         .
  205 AGGCACACAAATTTGACTACCCAA..........................
      ||||||||||||||||||||||||
72800 AGGCACACAAATTTGACTACCCAAAATGAGTGCCAACAACCCCTCATGTG
```

6.3.4 Luciferase activity assay

Initially, the experiment was conducted in a COS cell line. This is a fibroblastic cell line of monkey origin purchased from Qiagen. COS cells were transfected with the pGL2 basic and pGL2 control as well as the constructs. The results indicated that the ORF 50 promoter was not functional in COS cells although the SV40 promoter in pGL2 control was functional (result not shown). The experiments were therefore performed in BT cells, a bovine cell line in which the virus multiplies. The results of the luciferase assays are presented in Figures 6.3 (a-f) and are summarised in table 6.2.

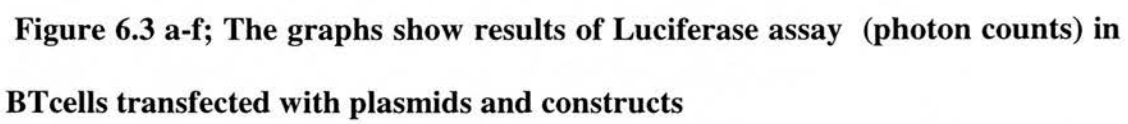


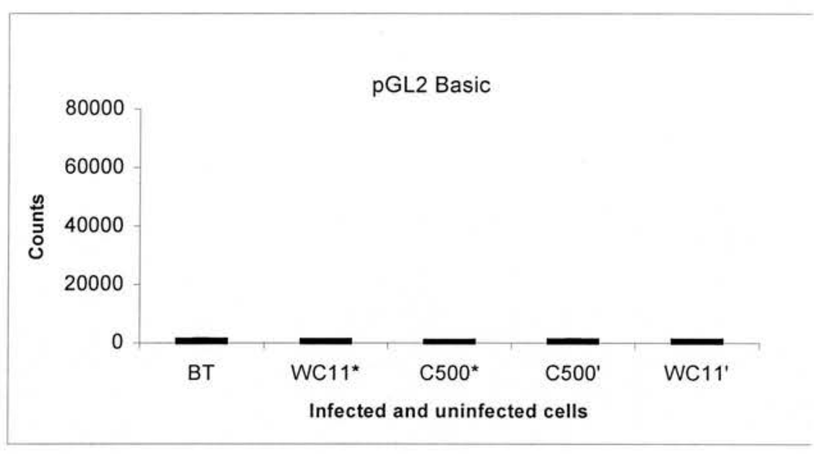
Figure 6.3 a-f; The graphs show results of Luciferase assay (photon counts) in BTcells transfected with plasmids and constructs

Indices; BT-uninfected cells,

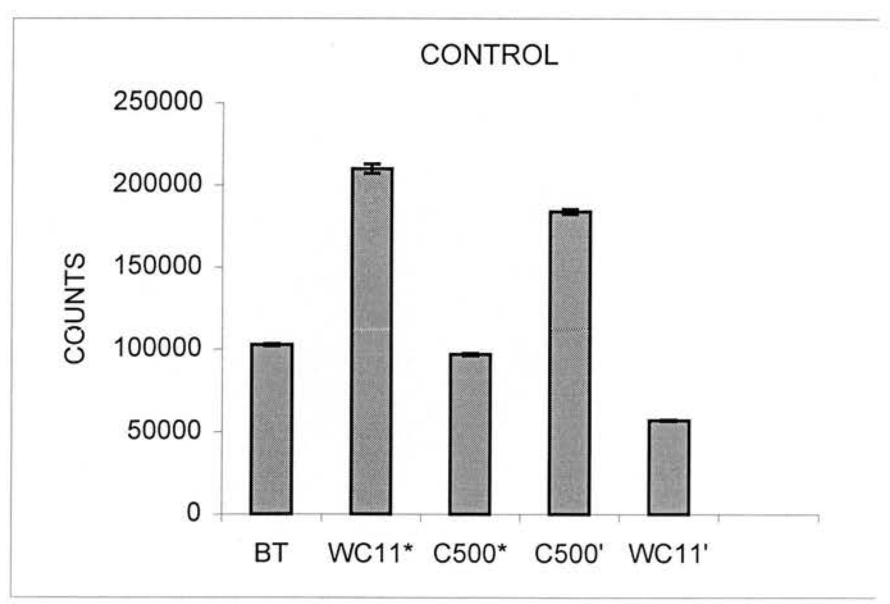
*- Cells infected with either virus before transfection,

'-BT cells that are transfected and then infected with the named isolate

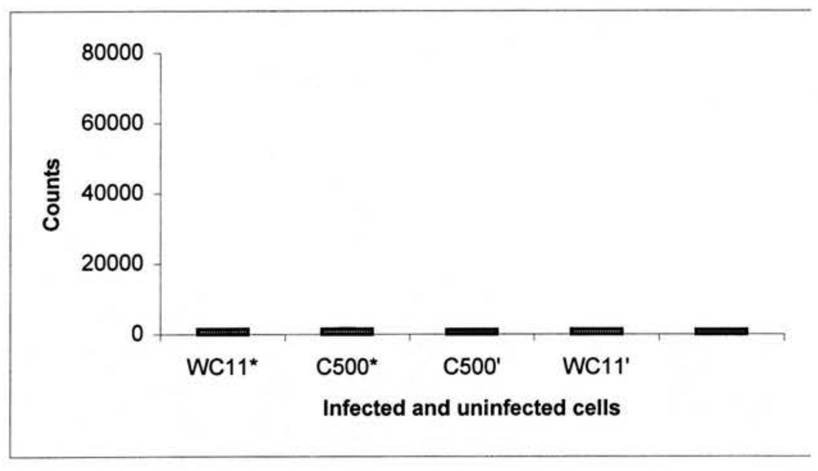
a) **Assay in BT cells transfected with pGL2 Basic**



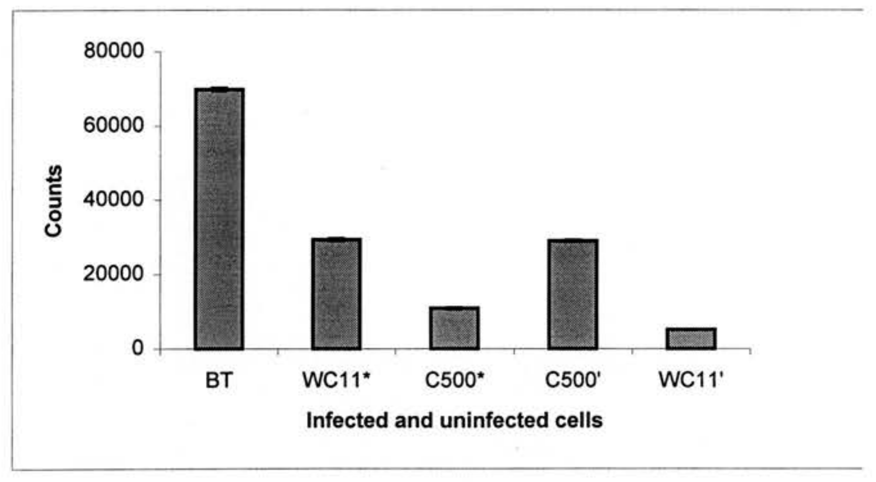
b) **Assay in BT cells transfected with pGL2 Control**



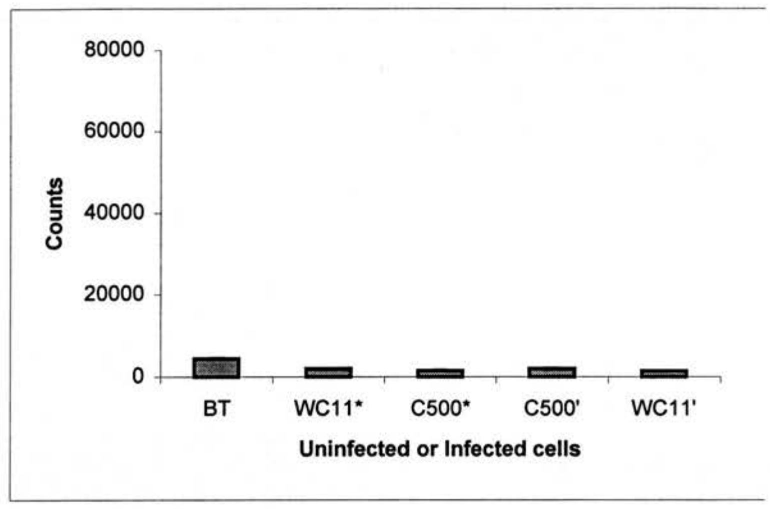
c) Assay in BT cells transfected with construct pGL2-CFA



d) Assay in BT cells transfected with construct pGL2-CA1



d) **Assay in BT cells transfected with construct pGL2-WC11**



f) **Assay in BT cells transfected with construct pGL2-CA2**

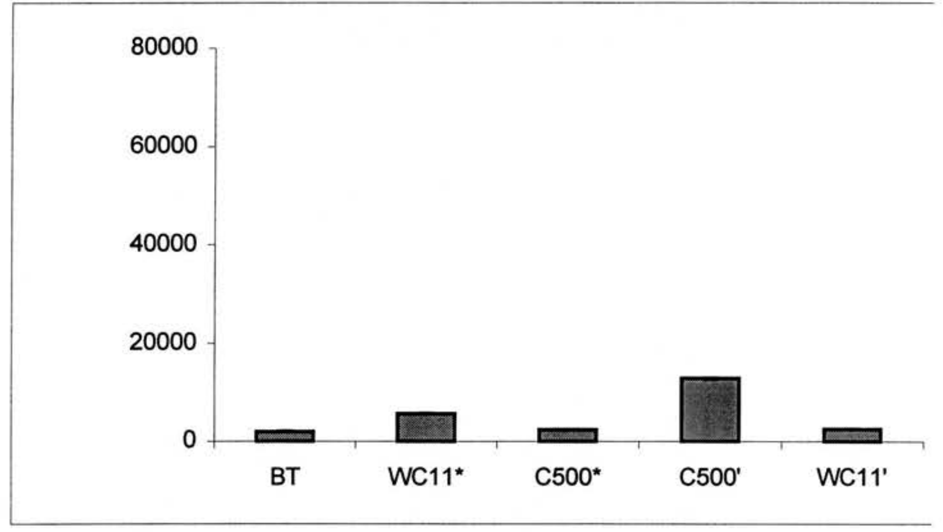


Table 6.2. Promoter activities of the pGL2-Control plasmid and the AHV-1 constructs relative to the promoter activity of the pGL2-Basic plasmid taken as one.

	Treatment of BT cells				
Plasmid	Transfection only.	Transfection followed by infection with the indicated virus.		Infection with the indicated virus followed by transfection.	
		C500 (CA)	WC11	C500 (CA)	WC11
Control	68.8	76.5	127.7	149.3	33.5
CA1	46.8	8.5	17.9	23.5	3.0
CA2	3.0	1.2	1.2	1.6	0.8
CFA	1.3	1.8	3.4	10.4	1.4
WC11	1.0	1.0	0.9	1.5	0.7
None	1.0	1.0	0.9	1.0	1.0

The promoter in the pGL2-control plasmid was activated to drive the luciferase gene in uninfected BT cells. The relative activity compared to pGL2-basic which lacks a promoter for the luciferase gene was 68.8 fold. When the assay was applied to the constructs, the ORF 50 promoter in pGL2-CA1 was functional with 46.8 fold the activity of the pGL2-basic plasmid. However, the activity of the constructs pGL2-CA2, pGL2-CFA and pGL2-WC11 were not sufficiently above background to be considered positive.

Reporter gene activity assay was also carried out on BT cells that were infected with either the virulent cell associated C500 or the attenuated WC11 24 hr after transfection to determine whether viral infection had an influence on the ORF 50

promoter activity. The enzyme activity in the cells transfected with pGL2-control increased almost two times to 127.7 fold that of the pGL2-basic plasmid in cells infected with WC11, but the activity was only slightly increased in cells infected with C500. The promoter activity of construct pGL2-CA1 was reduced to 17.9 fold in cells infected with WC11, and was reduced even further in cells infected with C500 to 8.5 fold. The ORF 50 promoter in the other constructs was not functional.

Assay in cells infected before transfection showed that the activity of the control plasmid was almost 150 and 33.5 fold in cells previously infected with C500 and WC11 respectively. In cells infected prior to transfection the promoter activity of pGL2-CA1 was reduced to 23.5 fold and 3 fold in cells infected with C55 and WC11 respectively. The ORF 50 promoter in the other constructs was not functional. No activity was demonstrated in any of the cell cultures transfected with the construct pGL2-CFA.

In all the cell cultures the cpe was extensive but the cells were still loosely attached to the cell culture flasks when they were harvested for assay.

6.4 DISCUSSION

The results demonstrate that the ORF 50 promoter construct pGL2-CA1 is functional in uninfected BT cells. pGL2-CA1 is a construct which contains 464 bp of DNA sequence derived from virulent C500 located immediately upstream of the ORF 50 start codon. Construct pGL2-WC11, which contains the 456 bp located upstream of the ORF 50 start codon but derived from the WC11 viral DNA was not functional, neither was pGL2-CA2 which contains a longer fragment, 950bp upstream of the start codon, derived from the virulent C500 viral DNA. It is possible that the longer

fragment may contain sequences that bind cellular repressors of ORF 50 promoter or cellular inhibitory factors. These inserts do however contain a consensus TATAA box at –274 bp relative to the ORF 50 ATG start codon. Another explanation for the inactivity of the ORF 50 promoter in pGL2-CA2 could be that the longer fragment may form secondary or tertiary conformational structures that make the promoter binding site inaccessible to the cellular transactivators.

Transient transfection assays were initially conducted in a COS cell line that is of monkey origin. There was no ORF 50 promoter activity in these cells although the pGL2-control SV40 promoter was activated to drive the luciferase gene. This indicated that these cells lacked a transactivator for the AHV-1 promoter suggesting that the ORF 50 promoter is activated by a specific transactivator. Zalani et al (1992) showed that the BRLF1 promoter in EBV, which is the equivalent of ORF 50 promoter could be activated by a transactivator, Sp1. Sp1 is an ubiquitous cellular transcription factor first described in HeLa cell cultures (Dynan and Tjian 1983) which has also been shown to transactivate the EBV BRLF1 promoter in transient transfection assays in epithelial cells but not in B cells. The authors suggested that in B cells the promoter may be negatively regulated by additional cellular factors. This transactivator binds to specific consensus binding sites, CCGCCC, CCACC and CCAAT. These Sp1 binding sites are present in BRLF1 promoter but are absent in the ORF 50 promoter. The ORF 50 promoter may therefore be transactivated by different proteins.

The results presented here provide further evidence that the putative ORF 50 promoter isolated from the attenuated C500 derivative (CFA) is non-functional. pGL2-CFA is composed of 274 bp identical to the sequence in AHV-1 (CA), at

positions 72550-72824 but inverted in orientation and ligated to 76 bp of sequence derived from H-DNA. The putative promoter is inactive probably because the transactivator does not recognise the inverted sequences, or the 274 bp fragment is too short to contain all the regulatory sequences required for activation of the ORF 50 promoter. It is also possible that the presence of H-DNA at the 3' end of the 274 bp sequence inhibits the binding of the cellular regulatory factor(s) for this promoter.

Interpretation of the results obtained in the experiments involving transfection/infection and infection/transfection is difficult for a number of reasons. Firstly, if the experiments were to be repeated, a co-transfected vector such as pCAT3 (Promega) which contains the chloramphenicol acetyltransferase reporter gene should be included to control for potentially variable transfection efficiencies. This does not affect the results discussed above where the differences between for example, pGL2-CA1 results for uninfected BTs and those for pGL2-CFA were too large to be accounted for by transfection efficiencies. However, discussion of the results where the differences were smaller is more problematical. Secondly, the way the experiment was set up, it must be borne in mind that we are dealing with an "immediate early" viral gene at "late" times i.e. 48 hours post infection when an "immediate early" promoter might not be expected to be activated. In the case of the transfection followed by infection experiments, this did not present a problem as the "immediate early" times began when the virus was added to the cells. The luciferase produced then may persist in the cells to be assayed 48 hours later. In the other experiments where the cells were infected and then transfected, the problem revolves around the question of multiplicity of infection and synchronicity of infection. If uninfected cells are still being infected when the transfections were occurring, then

clearly in those cells "immediate early" activity still prevails and the promoter may still be activated. Certainly, in the case of the virulent C500 strain, the virus is cell-associated and synchronicity of infection may be difficult to achieve.

Broadly, the results obtained in the infection and transfection assays demonstrate that for the pGL2-CA1 construct, the promoter function was reduced in virally infected cells, although for reasons discussed above it is difficult to be categorical. Certainly, in all experiments the level of activation of the promoters in the constructs other than pGL2-CA1 remained very low.

Generally speaking, the results are consistent with the observation that the gene for ORF 50 is transcribed in cell cultures infected with virulent virus and not in cells infected with the attenuated derivative of C500 (CFA) or WC11. Although transcription in herpesviruses is from either strand, the translocation and inversion of ORF 50 may be the reason for the trancriptional silencing of ORF 50. The other possibility may be that crucial regulatory sequences that are inverted following attenuation cannot be activated to drive transcription of ORF 50.

CHAPTER 7

GENERAL DISCUSSION

The first characterisation of the AHV-1 genome was conducted using viral DNA derived from the attenuated WC11 isolate (Bridgen et al, 1989). The genome was shown to comprise of up to 30 direct 1kbp repeats flanking a low G+C unique DNA (L-DNA) of approximately 130 kbp in length. The virus was therefore classified as a B- group gammaherpesvirus, the prototype of which is the Herpesvirus saimiri (HVS) (Roizman et al, 1992). The virulent pathogenic form is largely cell associated (CA), while the high passage cell free (CFA) derivative is non-pathogenic (Plowright, 1986). The genomes of these forms of the C500 virus isolate were compared by restriction enzyme analysis and southern blotting with a 3.8 kbp clone of CFA (Handley et al, 1995). Differences in the sma1 profiles of the two forms were described and three restriction fragments designated vir1, vir2 and att1 were cloned and sequenced. The clones were mapped to a terminal location in the genome based on their hybridisation to the ladder of restriction fragments which result from the variable number of the tandem repeat units at the end of the unique DNA. Differences in the nucleotide sequence were interpreted to be due to deletions which occurred on the rearrangement of the genome during the transition from virulence to attenuation. The results indicated that a fragment of approximately 500 bp was deleted in the attenuated genome. An analysis of the cloned DNA also indicated that there were nine putative open reading frames in these clones (Handley, 1993, Handley et al, 1995). The 500 bp fragment present only in vir2 was shown to encode a highly antigenic protein, designated polypeptide 5 (P-5). Comparisons with the HVS indicated that this putative protein would be the positional equivalent of the HVS transforming proteins STP A11 and STP C488 which are only present in the

virulent derivatives of strains A11 and C488 but are deleted following attenuation of these HVS sub-types (Albrecht et al, 1992, Beisinger et al, 1990). Another protein, present in both clones, and designated peptide 1 was shown to share limited homology to the Tyrosine Kinase Inhibitor Protein (TIP) in HVS (Murphy et al, 1989, Beisinger et al, 1990). Based on these suggestions, the aim of this study initially was to clone and express these putative proteins and assess their protective role and also determine whether these proteins are expressed *in vivo* and *in vitro* with the objective of using them as diagnostic reagents in AHV-1.

The results presented here show that these proteins were successfully expressed in *E.coli* and the recombinant proteins used to immunise rabbits. However, neither of the two proteins was protective although both proteins were immunogenic. P-5 cloned as a fusion protein with thioredoxin was evaluated as an antigen in an indirect ELISA and the results show that this protein is not recognised by sera obtained from cattle experimentally infected with either the virulent or attenuated virus. It was not possible to use peptide 1/GST fusion as the protein degraded very rapidly and use of the protease inhibitor, A-PMSF did not stabilise the protein. mRNA for both proteins was however detected in LCL derived from rabbits infected with either AHV-1 or OHV-2. This indicated that genes for both proteins are conserved in the two viruses. Both proteins were also detected in LCL as diffuse cytoplasmic antigen in an indirect immunofluorescence test. Both proteins were also detected in cultures infected with the virulent C500 and attenuated WC11. Detection of P-5 in cultures infected with WC11 was rather surprising as Handley et al (1995) indicated that the sequence encoding this protein is deleted in the attenuated virus. The attenuated virus used in

this study is the WC11 isolate whereas the att1 was derived from cell free attenuated C500. However, P-1 antigen detected in cells infected with WC11 was located in the nucleus whereas P-1 antigen was located in the cytoplasm and also in the nucleus in cultures infected with virulent virus. This may indicate that the sera was recognising different antigens in these cultures.

This study was carried out before the publication of the complete sequence of virulent C500 (Ensser et al, 1997). The sequence published is consistent with the findings of Bridgen et al. (1989) with regard to the organisation of the AHV-1 genome. The sequence also shows that the AHV-1 genome is generally collinear with that of other gammaherpesviruses. Ensser et al. (1997) have also described the potential open reading frames encoding genes for AHV-1. The C500 sequence shows that the genes encoding P-1 and P-5 were longer in size than described by Handley et al. (1995) and that these genes are located in the middle of the genome and not near the terminal repeats as previously noted. P-1 and P-5 would therefore represent parts of the ORFs 50 and A7 respectively. Although there were these two discrepancies with the Handley sequence in respect of these reading frames, the sequences which were cloned in these studies are in frame. The P-1 and P-5 fusion protein described here therefore represent parts of the proteins 50 and A7 respectively. These proteins could therefore represent antigenic epitopes in the ORFs described by Ensser et al. (1997).

It was however important to re-assess the sequences described by Handley et al. (1995) and Ensser et al. (1997) to confirm the sizes and positions of these ORFs in AHV-1. As the laboratory strains of AHV-1 have been propagated for many years,

two virus isolates were obtained from cattle showing clinical MCF and a further two isolates were also obtained from wildebeest. Viral DNA was extracted from the new isolates before they had undergone passage in culture. PCR using viral DNA from these new isolates with primers designed to amplify full length ORF 50 and A7 showed that fragments of predicted size were obtained. Sequences of the PCR fragments showed that the DNA was 100% homologous to C500 viral DNA. The results therefore confirm that the two ORFs were conserved between isolates. Restriction maps generated for ORF A8, which is adjacent to ORF A7 also showed that this ORF is also conserved.

This finding necessitated a re-assessment of clones vir1, vir2 and att1 to determine whether they were genuine clones or cloning artefacts. The Ensser et al. (1997) sequence had shown that although vir2 was a Hind III fragment derived from the middle of the genome, vir1 contained sequence from vir2 joined to the sequence from the 3' end of the genome around 129 kbp. PCR and sequence results presented here show that ORF 50, half of ORF A6 and 140 bp upstream of ORF 50 are translocated to a position adjacent to the terminal repeats in the attenuated virus. The sequences were mapped to the 3' end of the genome. It is therefore likely that the vir1 clone of Handley et al. (1995) was derived from the attenuated virus, probably because the virulent virus preparation contained a proportion of virus which had been rearranged.

Northern blot analysis of viral mRNA derived from cell cultures infected with the virulent and attenuated virus showed that ORF 50 is only transcribed in cultures infected with the virulent virus. In this study, luciferase was used as a reporter gene

to assay the activity of the ORF 50 promoter sequences in the attenuated and virulent virus. The results show that the promoter is functional in the virulent virus but is non-functional in the attenuated virus. Thus the transcriptional silencing of ORF 50 *in vitro* could be attributed to the inactivity of the promoter in the attenuated virus.

During the process of attenuation, not only are crucial ORF 50 promoter sequences truncated, but the regulatory sequences of ORF 48, a putative glycoprotein encoded on the (-) DNA strand, are also translocated to the 3' end of the genome. It has now been shown that ORF A6 is also truncated. Whatever the consequence of this rearrangement during the process of attenuation however, the virus productively replicates *in vitro.*

Most studies on the molecular basis of pathogenesis of gammaherpesviruses have been conducted with EBV (Rickinson and Kieff, 1996). EBV is a gammaherpesvirus that has been implicated as a cause of various forms of tumour and other lymphoproliferative conditions in humans (Kieff and Liebowitz, 1990, 1996). The virus latently infects and immortalises B lymphocytes such that the cells grow continuously in culture (Pope et al, 1968, Nilsson and Klien, 1982). Only a few of the approximately 100 genes of the virus are expressed when B cells are latently infected with EBV virus (Dambaugh et al, 1986). Some latently infected B cells however spontaneously enter the lytic phase of virus replication. Latently infected cells can also be induced to enter the lytic phase of virus replication with production of mature EBV particles using chemicals like 12-*O*-tetradecanoylphorbo-13-acetate (TPA) (Zur Hansen et al, 1978). During the lytic infection, immediate early (I.E.), delayed early (D.E.) and late (L) proteins are expressed. One I.E. protein, encoded by

BRLF1 is designated R in EBV (Hardwick et al, 1989). The equivalent of this protein in AHV-1 and HVS is encoded by ORF 50 (Ensser et al, 1997, Nicholas et al, 1991). Although this ORF is a positional equivalent of BRLF1, there is only limited sequence homology in the AHV-1 genome.

The BRLF1 gene in EBV is transcribed as a bicistronic mRNA which also encodes another immediate early protein, designated BZLF1. The Northern blot result presented here provides some evidence that ORF 50 mRNA in AHV-1 may also be bicistronic as the size of the mRNA which hybridises to a peptide 1 (ORF 50) probe is larger than expected. Both of these immediate early transcripts in EBV have been studied extensively and are now known to be critical in the induction of the lytic cycle of replication in latently infected cells *in vitro*. Expression of these proteins in B lymphocytes and epithelial cells latently infected with EBV can be induced by TPA (Zur Hansen et al, 1978). Initially R was shown to activate the delayed early gene, BZLF2, which codes for a protein that is an essential component of EBV viral DNA polymerase activity (Kenney et al, 1989). R protein together with Z are essential in activation of lytic replication in B cells latently infected with EBV but Z alone was sufficient to induce replication in latently infected epithelial cells (Kenney et al, 1989). This was an important finding as lytic replication occurs in epithelial cells before infection and establishment of latency in peripheral blood lymphocytes during EBV infection cycle *in vivo*. The proteins were shown to function by activating viral and cellular promoters leading to an ordered cascade of viral gene expression and release of mature virions (Parker et al, 1990). It has now been established that in EBV infection, R activates lytic cycle genes and can disrupt

latency in B lymphocytes (Ragoczy et al, 1998). The authors suggest a co-operation model for EBV entry into the lytic cycle. Expression of either R or Z triggers expression of the other I.E. factor and together these activators act individually or in synergy on downstream targets to activate the viral lytic cycle.

Although ORF 50 in AHV-1 shows positional homology but only limited structural homology to BRLF1, the gene products may perform the same function in cells infected by these viruses. This transactivator may be important in the establishment of latency in infected T cells. However, the attenuated virus which does not have a functional ORF 50 cannot establish latency *in vivo.* The protein was however detected in LCL derived from rabbits infected with AHV-1 and OHV-2. Productive replication does not occur in these cells (Reid-personal communication). Probably the establishment of latency is necessary in the pathogenesis of MCF. However mRNA for ORF 50 was not detected in peripheral blood lymphocytes obtained from cattle experimentally infected with either the virulent or attenuated AHV-1 using RT-PCR. In affected tissues from an animal with MCF only a few cells express AHV-1 antigen *in vivo* (Edington and Patel, 1981) and viral DNA can only be detected in 1/1000 infected T cells from clinically affected rabbits (Bridgen et al, 1992). The inability to detect ORF 50 mRNA may therefore be genuine or the result may reflect the sensitivity of the RT-PCR test.

The results presented here show that ORF 50 is translocated and its promoter truncated in the attenuated form of AHV-1. The overall effect of this is that ORF 50, a putative R transactivator is silenced *in vitro*.

The results also show that during the process of AHV-1 attenuation following passage of the virus in culture, sequence encoding a putative glycoprotein, A8 (Ensser et al, 1997) is truncated/translocated or deleted.

The putative glycoprotein of AHV-1 encoded by ORF A8 shows structural homology to gp350/220 encoded by BZLF3 in EBV. These are two glycoproteins that are transcribed from the same gene (Hummel et al, 1984, Beisel et al, 1985). Gp220 is spliced in frame but gp350 is translated from the un-spliced transcript (Hummel et al, 1984, Billaud et al, 1989).

In EBV this glycoprotein mediates attachment to B cells *in vitro* via the CR2 receptor on the surface on these cells (Fingeroth et al, 1984). CR2 is a type 2 complement factor that binds C3d component of complement. The glycoprotein mediates adsorption of EBV to CR2. The interaction may therefore mimic C3d binding and lead to receptor mediated endocytosis (Nemerow et al, 1985, 1987). Saturation of B cell receptors with anti-CR2 antibody blocks EBV adsorption, indicating that this receptor may be crucial in infection of B cells by EBV (Tanner et al, 1987, 1988). Entry of virus into cells in addition seems to require two other glycoproteins, gp85 and gp25 but the specific role that these latter glycoproteins play is not clear (Yaswen et al, 1993, Pulford et al, 1995).

The cotton tail tamarin (*Saguinus oedipus oedipus*), a laboratory model for EBV, has been shown to resist challenge with a lethal dose of EBV following vaccination with a replication defective EBV expressing purified gp350/220 (Ragot et al, 1993) and also with a subunit gp350/220 vaccine with adjuvant (Finerty et al, 1994) indicating that this glycoprotein plays a significant role in the pathogenesis of EBV.

In OHV-2 infection, the target cell in sheep is the B lymphocyte (Baxter et al, 1997). The lymphocyte sub-type associated with infectivity for AHV-1 in wildebeest is not known although the target cell for the virus in rabbit is the T-lymphocyte (Mushi et al, 1984). Although the CR2 is a receptor found only on the surface of B cells, this receptor has been found in a small proportion of human T- lymphoblastoid cells immortalised by Human T Cell Leukemia virus (Koizumi et al, 1992). These cells were successfully infected with EBV. It is possible therefore that a proportion of T cells in cattle and other susceptible species possess the CR2 receptor. No studies have yet been conducted on the adsorption and entry of cells during infection with AHV-1. This is difficult to investigate experimentally as only cell associated virus is available at primary isolation of the virus.

Virulent as well as attenuated AHV-1 are however able to replicate *in vitro*. This glycoprotein may therefore not be required for virus replication in culture. However, this receptor protein may be essential for virus transmission and replication *in vivo.*

This study has shown that genomic rearrangements occur following attenuation of AHV-1 with respect to ORF 50, ORF 48 and ORF A8. It has now also been shown that ORF A6 is also truncated. In the process of translocation of these genes, ORF A10 located at the 3' end of L-DNA is truncated.

Some of the pathological processes which characterise MCF may be precipitated by at least four bovid gammaherpesviruses. These include AHV-1 (Plowright et al, 1960), AHV-2 (Reid and Rowe, 1973), HipHV-1 (Reid and Bridgen, 1991), and OHV-2 (Reid et al, 1984). This suggests that the viral transcripts which precipitate the reaction are highly conserved. They must therefore have important biological

functions in the natural history of these viruses. The work described in this thesis has further characterised some of these candidate virus products.

The publication of the complete sequence of the AHV-1 genome provides an opportunity to study the putative genes, especially so those genes that may be important in elucidating the molecular basis of virulence in this enigmatic virus. Homologues to 60 conserved herpesvirus ORFs have been described. These include some putative genes that may be important in the pathogenesis of AHV-1. The mechanisms that probably play a role in the pathogenesis of MCF include those genes that may activate or suppress the immune system and also genes that may be responsible for the T-cell dysfunction. Genes that may have these roles in AHV-1 have now been described. Studies should be conducted on the role of ORF 57, a putative regulator of transcription to determine the role and function of this protein *in vivo* and *in vitro* and also on ORF A5, a putative G protein-coupled receptor that may modulate the host immune system and also deliver activation signals to transformed cells. Another open reading frame that may encode a putative determinant of virulence in AHV-1 is protein A9 that shares homology to the Bcl2 family of proteins that are regulators of apoptosis.

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Appendix 6.1. Location of the PCR primers used in this thesis at the left end, middle and right end of the AHV1 genome.

Boxes indicate the location of ORFs described by Ensser et al. 1997