MOLECULAR STUDIES OF ALCELAPHINE HERPESVIRUS 1 AND THE ROLE OF THE VIRUS IN MALIGNANT CATARRHAL FEVER.

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Contents.

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Abbreviations

AHV-1	*Alcelaphine herpesvirus* 1
ATP	adenosine triphosphate
bp	base pairs
BEB	bovine embryonic brain (cells)
BEK	bovine embryonic kidney (cells)
BET	bovine embryonic thyroid (cells)
BSA	bovine serum albumin
BT	bovine thyroid (cells)
cDNA	complementary DNA
Ci	curie
cpe	cytopathic effect
CsCl	caesium chloride
CSF	cerebrospinal fluid
DMF	dimethyl-formamide
DMSO	dimethyl-sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBV	Epstein Barr virus
EDTA	ethylene-diamine-tetra-acetic acid, disodium salt
FBS	foetal bovine serum
FTL	freeze-thaw lysate
HSV	Herpes simplex virus
HVA	*Herpesvirus ateles*
HVS	*Herpesvirus saimiri*
IIF	indirect immunofluorescence
IL-2	interleukin-2
IPTG	isopropyl-β-D-thio-galactoside
ISH	*in situ* hybridisation
kbp	kilobase pair
keV	kiloelectron volts
lmp	low melting point
LN	lymph node
MCF	malignant catarrhal fever
moi	multiplicity of infection
MRI	Moredun Research Institute
mRNA	messenger RNA
NK	natural killer (cell)
OD	optical density
OLB	oligo-labelling buffer
ORF	open reading frame
pcr	polymerase chain reaction
PEG	poly-ethylene glycol 6000
pfg(e)	pulsed-field gel (electrophoresis)
pi	post inoculation
pfu	plaque forming units
RNA	ribonucleic acid
rpm	revolutions per minute
SAA	sheep-associated agent (of MCF)
SA-MCF	sheep-associated MCF
SDS	sodium dodecyl-sulphate
SE	sonicated extract
SSC	standard sodium citrate
TCID	tissue culture infective dose
Tm	(duplex) melting temperature
uv	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

Abstract.

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative disease of cattle and deer. One causative agent is the gammaherpesvirus *Alcelaphine herpesvirus* 1 (AHV-1), which causes no disease in its natural host, the wildebeest *Connochaetes taurinus*. Epidemiological evidence also implicates sheep in the incidence of MCF. That the agent of sheep-associated MCF (SA-MCF) is a virus similar to AHV-1 is indicated by the detection of antibodies to AHV-1 in sera from sheep, from some cattle with SA-MCF and from hamsters experimentally infected with SA-MCF. The SA-MCF virus has never been isolated, but lymphoblastoid cell lines, some of which transmit the disease, have been propagated from cattle, deer and rabbits with SA-MCF.

The genome of the attenuated WC11 isolate of AHV-1 has been studied. It comprises a region of unique DNA of approximately 130 kbp and an additional 25-30 kbp of tandem repeat sequences. There is one major repeat sequence of 950 bp which is interspersed with a small number of related sequences of different length. 70% of the unique DNA has been cloned into the lambda vectors λEMBL4 and λNM1149, while the major repeat sequence was cloned into M13mp18. Restriction endonuclease analysis of these clones and of WC11 genomic DNA, combined with hybridisation of these clones to blots of digested WC11 DNA provided sufficient information to construct a restriction map for WC11 DNA with respect to *Bam*HI, *Eco*RI, *Sal*I, *Sma*I and *Xho*I. The restriction map indicated a terminal location for the repeat sequences. The genome of the virulent C500 isolate of AHV-1 was similar to that of WC11 in the unique region but possessed a single repeat sequence of 1050 bp.

WC11 clones were applied to the study of SA-MCF, using the lymphoblastoid cell lines derived from SA-MCF-affected animals. These cells showed no evidence of viral particles or antigens, but hybridisation experiments have shown that

five different unique region clones derived from totally different regions of the WC11 genome show homology with DNA extracted from these cell lines. However, no hybridisation was observed with the WC11 major *Hin*dII repeat sequence clone, M30. A genomic library of total DNA extracted from the cell line MF/629 was constructed in λNM1149 and two related clones isolated by hybridisation to a WC11 clone. These two putative SA-MCF viral clones were found to hybridise more strongly to DNA extracted from lymphoblastoid cell lines derived from SA-MCF -affected animals than to that derived from C500-affected animals.

There are conflicting theories for the pathogenic mechanisms of the malignant catarrhal fever viruses. Therefore sections from six organs taken from each of six rabbits experimentally infected with C500 were assessed for the presence of viral DNA by *in situ* hybridisation using the WC11 repeat sequence clone. Virus-infected cells were only detected in the lung of one rabbit and the popliteal lymph node of another, at levels of approximately $1:10^4$ cells. Lymphoid cells cultured from the lymph nodes of additional C500-infected rabbits showed no evidence of viral DNA prior to 12h of culture, but the number of virus-infected cells increased 10-100 fold following 12 to 36h of culture. It was concluded that AHV-1 must exert its effect by a means other than direct viral action.

This thesis thus describes the initial genomic characterisation of AHV-1, and shows how the WC11 clones were employed to demonstrate homology between the agent of SA-MCF and AHV-1 and to investigate the pathogenic mechanisms of AHV-1.

The Peak

We sailed in sunshine; but the glen was black
As Tartarus with raven clouds that swirled
In a fantastic frenzy, closely furled
One moment round the hills; now, streaming, torn
To ribbons; then in bundling fleeces whirled
As in a witch's cauldron, leaving bare
The jagged ranges to the pallid glare
Of lightning: and we heard the thunder crack
In short sharp volleys like quick rifle-fire:
Then once again the firth in instant night
Was blotted out; while still in lively light
We sailed serenely on through the blue morn
Toward the islands of our heart's desire.

But, ere we lost the land, a brooding cloud
On the horizon, suddenly the shroud
Slipped from the shoulders of a single peak
That soared in sunshine like a soul set free
Of the gross turmoil of mortality:
And, as we gazed, our hearts, too full to speak,
Found in that vision all we sailed to seek.

Wilfrid Gibson.

CHAPTER 1

INTRODUCTION.

1.0 Introduction.

This chapter begins with a discussion of malignant catarrhal fever (MCF) to illustrate the biological properties of *Alcelaphine herpesvirus* 1 (AHV-1), the main subject of this thesis. This is followed by an introduction to the genomic organisation and biological properties of the herpesviruses in general and the gammaherpesviruses in particular, since it is to this group that AHV-1 shows the greatest similarity. The chapter ends with a discussion of the molecular characterisation of AHV-1 which had been performed at the start of this study.

1.1 Malignant catarrhal fever (MCF).

Malignant Catarrhal Fever or "Snotsiekte" is a fatal lymphoproliferative and degenerative disease of *Bovidae* and *Cervidae*. The disease has been recognised in cattle for more than one hundred years (Goetze, 1930) and in deer for more than thirty years (Tong *et al.*, 1961; Huck *et al.*, 1961 and Senior *et al.*, 1962) although the first reported case of MCF in deer may in fact precede that in cattle (Lupke, 1906). There have been reported cases of MCF in at least forty species of ruminant located throughout the world, either in free-living animals or in those captive in zoos or animal parks (Reid and Buxton, 1984 and summarised by Heuschele *et al.*, 1988). These include species located in Europe, America, Africa and Asia. Several economically important Asian animals including the water buffalo (*Bubalus bubalis*) and Bali cattle/Banteng (*Bos javanicus* synonym *Bos sondaicus*) are particularly susceptible (Hatkin, 1980; Daniels *et al.*, 1988). The importance of MCF has also increased with the development of deer farming in the UK, New Zealand and Australia (Reid *et al.*, 1979; Oliver *et al.*, 1983 and Denholm and Westbury, 1982). MCF generally occurs sporadically but epizootics have been observed in cattle, deer and bison (Pierson *et al.*, 1973; Reid *et al.*, 1979 and Ruth *et al.*, 1977).

The viral aetiology of the disease is complex, with at least two causative agents. The gammaherpesvirus *Alcelaphine herpesvirus* 1 (AHV-1) is carried by apparently healthy wildebeest (*Connochaetes taurinus*) (Plowright, 1968) and similar viruses have been isolated from other African antelopes (Reid and Rowe, 1973; Mushi *et al.*, 1981 and Seal *et al.*, 1987). There is increasing evidence that domestic sheep harbour a virus similar to AHV-1. This virus has not been isolated, and is therefore described in the literature as the sheep-associated agent (SAA) of MCF. The aetiological agents are discussed further in Section 1.1.2.

1.1.1 Clinical features of the disease.

MCF is an acute, subacute or chronic disease characterised by high fever of up to 42°C and severe inflammatory and degenerative lesions in the mucosae of the upper respiratory and alimentary tracts. The pathological changes induced by the different aetiological agents are similar but the most comprehensive studies have been on AHV-1-induced disease. The following description therefore refers to AHV-1 -induced pathological changes. Enlargement of lymphoid organs and lymphoid accumulations in many tissues occur in the majority of cases. Conjunctivitis and corneal opacity frequently occur, the latter of which may lead to blindness. There are often lesions of the skin, gums and muzzle, and perturbation of the central nervous system. (Plowright, 1968). The incubation period is approximately twenty days in cattle following experimental inoculation (Plowright, 1982), but varies with the aetiological agent and with the dose inoculated (Pierson *et al.*, 1979 and Buxton *et al.*, 1984). Viraemia, which is associated with lymphocytes, is usually first detectable from 0 to 7 days before the onset of pyrexia and increases to a maximum at the onset of pyrexia (Plowright, 1982). Death occurs beween five and twelve days later in 95% of cases (Plowright, 1968) but persistent infection can occur (Rweyemamu *et al.*, 1976). Plowright *et al.*, 1972 described one experimentally-infected cow showing no symptoms of MCF

which produced four viraemic calves. However, some authors consider there to be no true cases of recovery from at least the SAA-induced form of MCF (SA-MCF) (Selman, 1987). Post mortem examination of infected cattle reveals enlarged, haemorrhagic and oedematous lymph nodes, and lesions of the urinary, respiratory and gastro-intestinal tracts. Lesions of the kidney, brain, urinary bladder and buccal epithelium are most useful for diagnostic purposes. (Reid *et al.*, 1984b and Reid, unpublished). Some authors distinguish four clinical forms of the disease:

1) a peracute form, with early mortality (1-3 days)
2) head and eye form, mortality 1-3 weeks
3) intestinal form, mortality around 9 days
4) benign

(Goetze, 1930). However, there seems to be no correlation between particular outbreaks and any one form (for example Pierson *et al.*, 1973).

Deer tend to develop a peracute form of the disease. Symptoms include high temperature, diarrhoea and dysentery which may lead to dehydration, and haemorrhagic and necrotic lesions of the gastro-intestinal tract (Reid *et al.*, 1984b). Deer are very susceptible to MCF, and experimental transmission of both AHV-1- and SAA- induced MCF to deer is relatively easy (Buxton and Reid, 1980 and Westbury and Denholm, 1982)

The pathology of MCF in Bovidae and Cervidae of the SAA and AHV-1 is similar, but there are differences in the incubation periods, ease of transmission and pathology (Plowright, 1968; Selman *et al.*, 1974 and Pierson *et al.*, 1979). Slight differences in the pathology occur in SAA and AHV-1 -infected rabbits. The latter experience a much greater T cell proliferation in peripheral lymph nodes and spleen, and a lesser response in mesenteric lymph nodes (Buxton *et al.*, 1984).

1.1.2 Aetiology.

1.1.2.1 Alcelaphine herpesvirus 1 (AHV-1).

In Africa, infections in cattle often follow association with apparently normal wildebeest (Mettam, 1923). There are approximately two million wildebeest in Africa, both the South African black wildebeest also known as the white-tailed gnu, *Connochaetes gnu*, and the East African blue wildebeest, *C. taurinus*. It is the blue wildebeest which has been studied most extensively for its involvement in malignant catarrhal fever. Blue wildebeest are migratory, moving between Northern Tanzania and Southern Kenya. Wildebeest calve between January and March in Northern Tanzania, and between February and April in Southern Kenya and the incidence of MCF is highest in these two areas in March-April and April-June respectively (Plowright, 1982).

A herpesvirus was isolated from blue wildebeest which was capable of inducing MCF on inoculation of susceptible animals (Plowright *et al.*, 1960 and 1963). Thus the virus, designated *Alcelaphine herpesvirus* 1 (AHV-1), was implicated as the primary causative agent of malignant catarrhal fever in East Africa. The carrier status of wildebeest with respect to MCF was confirmed by the isolation of AHV-1 from peripheral blood leucocytes and from splenic and other lymphoid tissue from some apparently healthy wildebeest of all ages (Plowright, 1965).

The epidemiology of infection in wildebeest in East Africa has been studied extensively. A small percentage of calves are viraemic at birth, having been infected *in utero* (Plowright, 1965). Calves acquire maternal antibodies to AHV-1 through colostrum but this does not appear to prevent infection. The antibody titres decline over a period of weeks with decline of maternal antibody but rise again following infection with AHV-1 (Plowright, 1967). It is likely that ocular and nasal secretions of young calves are the normal route of excretion, since virus has been

isolated from these secretions (Mushi *et al.*, 1980). Older calves possess AHV-1 -neutralising IgA in nasal secretions which presumably prevents transmission after this age (Rurangirwa *et al.*, 1982). All wildebeest calves over nine months possess serum neutralising antibodies against AHV-1 but show no clinical symptoms. However, beta-methasone treatment of adult wildebeest was found to cause a reactivation of virus and further excretion in nasal fluids (Rweyemamu *et al.*, 1974) and it is likely that other stress factors may cause a similar reactivation.

The first well-characterised AHV-1 isolate from blue wildebeest to be described was designated WC11. This isolate produced cell free virus following forty-nine passages in calf kidney cells (Plowright *et al.*, 1963). Although initially virulent, this isolate has now become attenuated for cattle (Plowright *et al.*, 1975). A virulent, cell-associated isolate designated C500 was desc-ribed by Plowright *et al.* (1975). An electron micrograph of WC11 virions is shown in Fig. 1.1.2.1.

1.1.2.2 The sheep-associated Agent (SAA).

MCF also occurs sporadically where cattle are in contact with sheep, particularly lambing flocks. No causative agent has been identified in sheep. Several viruses have been isolated from affected cattle including herpesviruses (Hambdy *et al.*, 1978; Storz *et al.*, 1976); bovine syncytial viruses (Storz *et al.*, 1976; Clarke *et al.*, 1973) a morbillivirus (Coulter and Storz, 1979), an enterovirus (Storz *et al*, 1976), a parvovirus (Storz *et al.*, 1976) and a togavirus (Clark and Adams, 1976). The presence of these viruses is probably fortuitous since inoculation of susceptible animals with these viruses has not led to the development of MCF (Reid and Buxton, 1984). The herpesvirus described by Hamdy *et al.* (1978) was presumably a laboratory contaminant since it was quoted in the literature 10 years later as an AHV-1 isolate (Rossiter *et al.*, 1988).

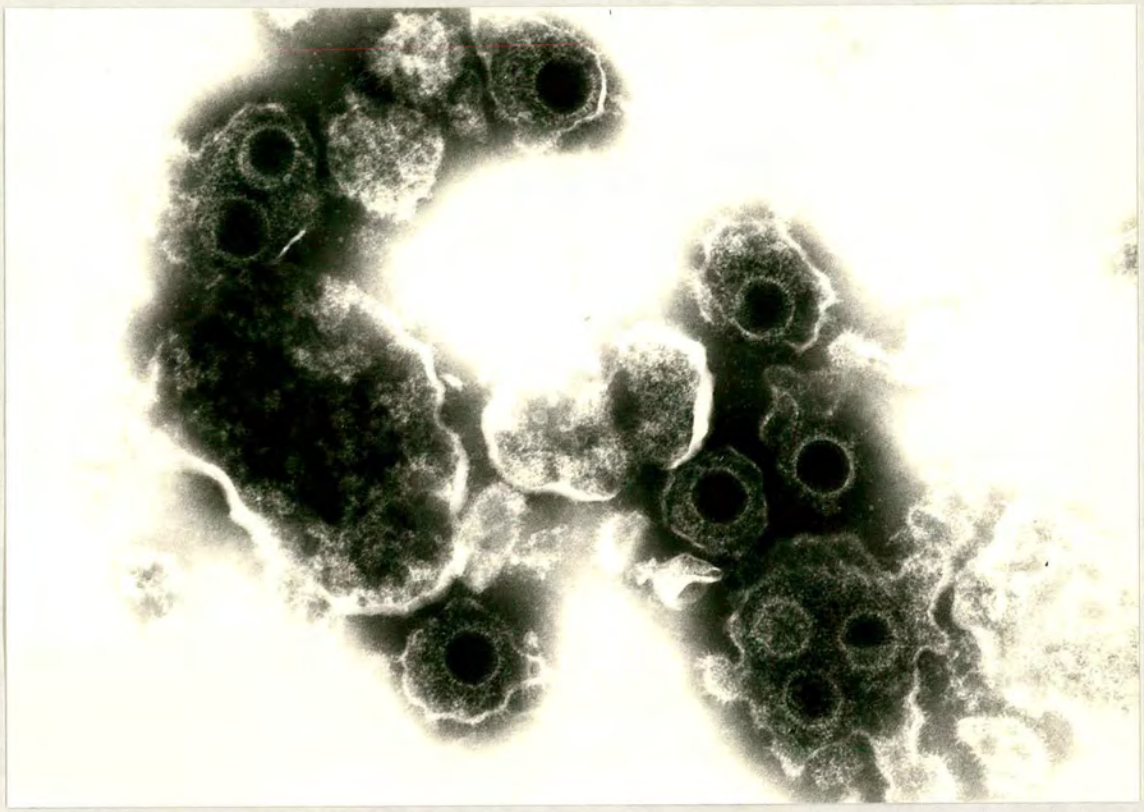


Figure 1.1.2.1
Electron micrograph of WC11 virions, magnification 80,000 times.

Note the presence of two nucleocapsids within one envelope. This is a feature commonly observed with AHV-1, but it does occur in other herpesviruses (Roizman, 1969).

There is increasing evidence that domestic sheep harbour a virus which is antigenically related to AHV-1. Rossiter (1981) found antibodies against AHV-1 in 162/167 sera from sheep taken from five continents using indirect immunofluorescence (IIF) and proposed that there was a similar but not identical virus prevalent in sheep. These experiments were extended to cattle putatively infected with the SAA and 38/48 naturally infected animals were shown to have significant titres of IIF antibody against AHV-1 (Rossiter, 1983). Immunoblotting experiments have shown that ovine and wildebeest sera and some bovine sera recognise antigens of the WC11 isolate of AHV-1. WC11 antigens were also recognised by components of bovine sera taken from animals experimentally infected with AHV-1 or from natural cases of SA- MCF. (Herring *et al.*, 1989). Finally, hamsters experimentally infected with SA-MCF develop antibodies which react with AHV-1 -infected cells, but not uninfected cells (Reid *et al.*, 1989a).

1.1.2.3 Other causal agents.

The presence of AHV-1 -like viruses in African antelopes other than wildebeest was suggested by the detection of neutralising antibodies to AHV-1 in Coke's hartebeest (*Alcelaphus buselaphus cokei*), topi (*Damaliscus korrigum*) and fringe-eared oryx (*Oryx beisa callotis*) (Reid *et al.*, 1975, Barnard, 1985; Hamblin and Hedger, 1984). These antelopes belong to the *Alcelaphinae* (hartebeest and topi) and *Hippotraginae* subfamilies; some of the authors described neutralising antibodies in members of the *Tragelaphinae* and *Antilopinae* subfamilies also, for example in the Greater Kudu (*Tragelaphus strepsiceros*) and Impala (*Aepyceros melampus*) respectively. The antibody titre in all these species was found to be independent of the proximity of wildebeest; therefore it was concluded that antigenically related but distinct viruses circulate in these species. A virus designated K30 was isolated from a hartebeest by Reid and Rowe (1973) which produced typical MCF when inoculated

into cattle (Reid, personal communication). Both this virus and that isolated from topi by Mushi *et al.* (1981) were difficult to grow in culture, preventing further analysis; it therefore was not conclusively proven that these viruses were different from AHV-1.

There is increasing molecular evidence to suggest that there are different viruses in different species of antelope. Heuschele's group used restriction endonuclease analysis and DNA hybridisation to confirm that viruses isolated from hartebeest and topi were very similar, and that they were markedly different from WC11. A virus isolated from a kudu did not hybridise strongly to either WC11 or topi genomic DNA (Seal *et al.*, 1987). The hartebeest virus has therefore been designated *Alcelaphine herpesvirus* 2, AHV-2 (Roizman, 1982).

1.1.3 Transmission experiments.

Natural transmission of MCF is believed to occur only from wildebeest or sheep and not from clinically affected animals. The susceptibilty varies both with the species and the circumstance, for example cases of SA-MCF regularly occur in farmed Red deer but no cases have ever been described in free-living Red deer (Reid, personal communication).

Experimental transmission of AHV-1 -induced MCF to cattle and rabbits has been reported by several groups (Daubney and Hudson, 1936 and Piercy, 1955). More recently, transmission from cattle to deer (Whitenack *et al.*, 1981) and from rabbits to rats, hamsters and guinea pigs has been achieved (Reid and Buxton, 1985; Jacoby *et al.*, 1988a and b). Transfer was again usually performed using single cell suspensions of mesenteric lymph nodes. SA-MCF has been transferred erratically to cattle, several species of deer, bison, rabbits and hamsters, but with more difficulty than for the AHV-1 -induced disease. (Selman *et al.*, 1978; Buxton

and Reid, 1980; Liggitt *et al.*, 1980; Westbury and Denholm, 1982; Reid *et al.*, 1986 and Jacoby *et al.*, 1988a). Few of the transmission steps are reversible, suggesting that some component of the virus may have been lost on heterologous transmission (Reid and Buxton, 1985). SA-MCF has been transmitted to rabbits using lymphoblastoid cell lines derived from MCF-affected animals (Reid *et al.*, 1985 and 1989b); these cell lines are discussed further in chapter 5.

1.1.4 The detection of viral antigen in AHV-1 -affected animals.

The small amount of published work on the detection of viral particles and antigens in MCF-affected animals has all been performed on animals affected with AHV-1 and indicates that there is very little antigen expressed. Rossiter (1980) assessed frozen sections of tissues taken from 6 experimentally-infected rabbits and 2 calves by direct immunofluorescence using hyperimmune rabbit and ox serum. Only rare, isolated fluorescent cells were seen in sections of lymphoid tissues at a frequency of 1 or 2 cells per 7 or 8 sections. Use of complement fixation, immunodiffusion and counter-immuno electrophoresis failed to reveal viral antigens in the same tissues, but infectivity was readily demonstrated in tissue culture.

Patel and Edington (1980) used indirect immunofluorescence (IIF) to detect AHV-1 antigens in rabbit lymphocytes. Both viral infectivity determinations and the IIF experiments provided an estimate of 1-4 virus-infected cells per 10^6 cells in a cell suspension. Following 2 -3 days *in vitro* culture the number of virus -infected cells increased by a factor of 50-1000. In addition, 40-100 fold more infected cells were observed in rabbits killed on the second or third day of pyrexia as opposed to the first day. The virus-infected cells appeared to be medium sized lymphocytes of 10-13 um diameter; electron microscopic studies confirmed the presence of viral particles within these cells. Similar results were obtained for infected

cattle (Patel and Edington, 1981), but there was a smaller increase in the number of infected lymphocytes on *in vitro* culture, although the initial infectivity of these cells was higher than in rabbits.

Edington and Patel (1981) extended these observations in order to determine the location of primary replication of AHV-1. Rabbits inoculated with a lymph node suspension taken from a MCF-affected rabbit were killed during the incubation period at 2, 4, 6 and 8 days post inoculation (p.i). The remaining 2 rabbits died at 12 days p.i. Virus was isolated from the spleen of 2/3 rabbits killed on day 4, and antigens were detected in the thymus and in the popliteal and retropharyngeal lymph nodes of these animals by IIF. No virus was isolated in any of the rabbits killed on day 6 and the only positive IIF results were for the spleen. The rabbits killed on day 8 showed more evidence for the presence of virus while the rabbits which died at 12 days p.i. were positive for virus in all tissues by infectivity and IIF. The authors concluded that the spleen was therefore the site of primary replication. However, the possibility of the virus detected being a part of the initial inoculum was not discussed.

1.1.5 The pathogenesis of MCF.

Several mechanisms have been proposed to account for the tissue necrosis and lymphoid accumulation induced by AHV-1. These are as follows:

1) Viral induced cytolysis (Liggitt and deMartini, 1980a)
2) Viral induced transformation (Edington *et al.*,1979 and Hunt and Billups, 1979).
3) Antibody induced immune complex formation or cytolysis of virus-infected cells (Plowright, 1968).
4) Antibody or cell mediated cytolysis of virus-infected cells (Selman *et al.*, 1974 and Liggitt and deMartini, 1980b).
5) Autoimmunity (Reid and Buxton, 1984).

The first mechanism is unlikely to be true, since viral particles and antigens are not found within the necrotising tis sues as discussed above. The oncogenic mechanism was suggested on the grounds of similarity in biological properties to the oncogenic herpesvirus *Herpesvirus saimiri*, HVS, for example in the proliferative nature of the lesions, the lack of contagiousness, variable incubation periods and the cell-associated nature of the virus. However, there is no convincing evidence in support of this hypothesis. No antigen-antibody complexes have been noted in support of mechanism 3. Insufficient antigen has been detected in the tis-sues of affected animals for support of mechanism 4. An autoimmune mechanism thus presents an attractive alternative explanation.

The pathogenesis of the MCF agents has been studied using SA-MCF -affected rabbits (Buxton *et al.*, 1984). Experimentally infected rabbits all showed a lymphocyte proliferation starting from a few days after inoculation with the SAA. The distribution of the lymphoproliferation to regions such as the paracortical areas of lymph nodes and the thymus indicated that T cells were responsible and this was confirmed by the use of T- and B- specific immunological markers. The T cell proliferation was not in itself the cause of death, since treatment of infected rabbits with the T lymphocyte inhibitor cyclosporin-A prevented T cell proliferation, while not affecting the incubation period or the lethality. These results and the normal architecture of the lymph nodes of MCF-affected rabbits suggested that viral-induced transformation of lymphocytes is not the basis of action of the SA-MCF agent.

T lymphocytes and at least one other cell type have been implicated as target cells for the MCF viruses. Lymph node suspensions taken from MCF - affected rabbits were separated into T cell enriched and depleted fractions; both fractions transmitted MCF but transmission of the T cell enriched fraction was destroyed by anti-T antiserum. This suggested that T lymphocytes and at least one other cell type could transmit the disease (Reid and Buxton, 1984).

Large granular lymphocytes (LGLs) with the properties of natural killer (NK) cells have been implicated as additional target cells (Reid *et al.*, 1983). NK cells are lymphoid cells important in destroying malignant and virally infected cells in a non-specific manner before specific immune mechanisms are mobilised. They were first implicated by their detection in the supernatant fluid from foetal ovine kidney cells co-cultivated with lymphocytes from an MCF-affected rabbit. After twenty-two days of culture the monolayer degenerated and many cells were present in the supernatant, which resembled LGLs in both morphological and histological properties. These cells, designated MF/120, could be cultured *in vitro* and killed a wide range of target cells in cytotoxic assays including foetal ovine kidney, bovine embryonic kidney, baby rabbit kidney, rabbit kidney and baby hamster kidney cells. The MF/120 cells had a rabbit karyotype. This was determined since the cell fusion -promoting agent polyethylene glycol had been employed at the initiation of culture. The cells could transmit MCF to rabbits following parenteral inoculation. Viable MF/120 cells were necessary for disease transmission. Cells killed by freeze-thaw or treatment with anti- T lymphocyte antibodies did not transmit infection. (Reid *et al.*, 1983).

Cells with the characteristics of LGLs have subsequently been cultured from a wide range of tissues derived from SA-MCF affected animals including: bovine cerebro-spinal fluid (CSF), lymph node and cornea, red deer CSF, lymph node and thymus, rabbit lymph node and spleen and rat spleen. These lymphoblastoid cell lines show NK-like activity (Reid *et al.*, 1984a, 1985 and 1989b). NK-like activity has also been observed in SA-MCF -affected rabbits, and the level of NK-like activity increased dramatically during the first three days after the onset of pyrexia. In contrast, negligible NK activity was detected in suspensions of lymph node cells from normal rabbits (Reid and Buxton, 1984). The LGLs often possess T-cell markers (Reid, personal communication), so the observation that there are two cell types which transmit MCF may not, in fact, be correct.

Although histologically and morphologically resembling NK cells, the cells isolated from MCF-affected animals do not fulfill the recent definition of human NK cells as $CD3^-$ T cell receptor (alpha, beta, gamma, delta)$^-$ LGLs which commonly express CD-16 and NKH-1 (Leu-19) cell markers and which mediate cytolytic reactions independent of the expression of MHC class I or II molecules on the target cells (Ades and Lopez, 1989). Most of the studies on cytotoxic cells isolated from MCF-affected animals have been performed using cattle, for which the cell markers are well characterised. It has been proposed that the NK-like cells cause the symptoms of MCF by secreting the T cell growth factor interleukin 2 (IL-2) to stimulate T cell proliferation and by extending their normal cell killing to a wide range of normal tissues (Reid and Buxton, 1985). This hypothesis is consistent with the widespread tissue necrosis and presence of NK-like cells only in the terminal stages of the disease.

1.1.6 Protection experiments against MCF.

Numerous cases have been reported in which neutralising antibodies against AHV-1 were present in susceptible animals, but these antibodies did not aid recovery from MCF or prevent infection from further challenge with AHV-1 (Plowright *et al.*, 1975; Rossiter, 1982a). It is thought that maternal antibodies in wildebeest calves do not prevent infection since viraemia and neutralising antibodies can often be detected simultaneously (Plowright, 1967).

Several authors have obtained partial protection against AHV-1. Russell (1980) found that vaccination of cattle with cell free C500 (C500 cf) inactivated by ultrasonic treatment or by acetylethyleneimine provided partial protection against challenge with C500 cf, while Edington and Plowright (1980) found that vaccination of cattle and rabbits with inactivated C500 provided protection from challenge with cell-free but not cell-asociated virus. Rossiter (1982b) found that WC11 -infected bovine cells

failed to protect rabbits against challenge with C500 -infected rabbit spleen cells despite the presence of viral neutralising antibodies. However, WC11-infected rabbit cells provided protection against a similar challenge at 47 but not at 90 weeks post vaccination. The success in the second experiment was attributed to either use of rabbit cells or to the higher level of neutralising antibody as a result of additional vaccinations; it should be noted that the level of neutralising antibodies had dropped substantially by 90 weeks. Remarkably, some protection against AHV-1 infection was provided by BHV-4 (Rossiter *et al.*, 1988. The authors refer to the virus as BHV-3 but see Section 1.2 below). The mechanism of this protection was unknown, and cross-reactivity or interference was discussed.

1.2 Classification of AHV-1.

The Herpesvirus Study Group of the International Committee for the Taxonomy of Viruses recommended a binomial system as follows:

i) The first name should reflect the family (when the name should end in *-id*) or subfamily (when it should end in *-ine*) of the primary host.

ii) The second name should be herpesvirus.

iii) Different herpesviruses with the same primary host should be sequentially numbered using Arabic numbers.

(Roizman, 1982).

Thus the subject of this thesis, commonly known as Malignant Catarrhal Fever Virus (MCFV), is also known as *Alcelaphine herpesvirus* 1, since its natural host is the African blue wildebeest, *Connochaetes taurinus*. The wildebeest is a member of the family *Bovidae*, and sub-family *Alcelaphinae*, comprising hartebeest, blue and black wildebeest, topi, tsessebe, blesbuck and Hunter's antelope (Bouliere, 1983).

AHV-1 also appears in the literature as a bovine herpesvirus. The classification varies with the author, but it is usually classified as *Bovid herpesvirus* 3 (BHV-3) (for example Ludwig, 1983). Although formally correct, the family *Bovidae* comprises 45 genera and 110 species, so classification according to the subfamily of the natural host is somewhat more meaningful. The classification of the bovine herpesviruses in general is confused, particularly for the bovine cytomegaloviruses, which have been classified as BHV-3 and as BHV-4 (summarised by Bartha *et al.*, 1987). This thesis follows Ludwig (1983) in the classification of the bovine herpesviruses, since most recent literature on bovine cytomegalovirus classifies this virus as BHV-4, and therefore researchers in this field have proposed that the virus is always given this name (Bartha *et al.*, 1987). Thus, BHV-1 is the alpha herpesvirus also known as infectious rhinotracheitis virus; BHV-2 is the alphaherpesvirus responsible for bovine herpes mammilitis; BHV-3 is AHV-1; BHV-4 is a betaherpesvirus responsible for a range of respiratory disorders in cattle; BHV-5 is the herpesvirus associated with sheep pulmonary adenomatosis (also known as *Caprine herpesvirus* 1, Roizman, 1982) and BHV-6 is a goat herpesvirus (also known as *Caprine herpesvirus* 2, Roizman, 1982 or *Caprine herpesvirus* 1, Engels *et al.*, 1987).

More than eighty species of herpesvirus have now been isolated from reptiles, amphibia, birds and mammals. These vary greatly in their biological properties and in the size, base composition and arrangement of their genomes (Roizman, 1982; Honess and Watson, 1977 and Honess, 1984). Herpesviruses therefore tend to be additionally classified according to these properties.

1.2.1 Classification of AHV-1 according to its biological properties.

Herpesviruses have been classified into alpha-, beta- and gamma- subfamilies on the basis of the host range, cytopathology, the duration of the reproductive cycle and

Alphaherpesviruses

Host range

In vivo variable from very wide to very narrow; *in vitro* also variable.

Cytopathology

Rapid spread of infection in cell culture resulting in mass destruction of susceptible cells. Establishment of carrier cultures of susceptible cells harbouring nondefective genomes difficult to accomplish.

Latent infections

Frequently, but not exclusively, in ganglia.

Examples

HSV-1, HSV-2, Equine abortion virus, pseudorabies virus.

Betaherpesviruses

Host range

In vivo narrow, frequently restricted to the species or genus to which the host belongs; *in vitro* replicates best in fibroblasts, although exceptions exist.

Duration of reproductive cycle

Slowly progressing lytic foci in cell culture. The infected cells frequently become enlarged (cytomegalia) both *in vivo* and *in vitro*. Inclusions containing DNA are frequently present in both nuclei and cytoplasm. Carrier cultures are easily established.

Latent infections

Possibly in secretory glands, lymphoreticular cells, kidneys and other cells.

Examples

Cytomegaloviruses of man, mice, pigs and guinea pigs.

Gammaherpesviruses

Host range

In vivo limited to the same family or order as the host it naturally infects. *In vitro* all members of this subfamily replicate in lymphoblastoid cells, and some also cause lytic infections in some types of epithelioid and fibroblastoid cells. Viruses in this group are usually specific for either B or T lymphocytes. In the lymphocyte, infection is usually arrested either at a prelytic stage with persistence and minimum expression of the viral genome or at a lytic stage causing cell death without production of complete virions.

Duration of reproductive cycle

Variable.

Cytopthology

Variable.

Latent infections

Latent virus is frequently demonstrated in lymphoid tissue.

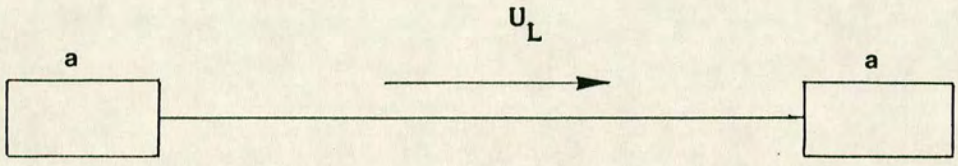
Examples

EBV, Marek's disease virus.

Table 1.2.1.1
Biological classification of the herpesviruses (Roizman, 1982).

Group Genomic organisation

A



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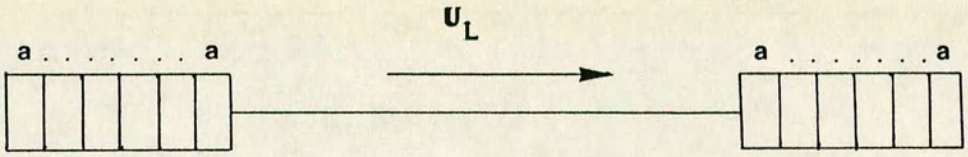

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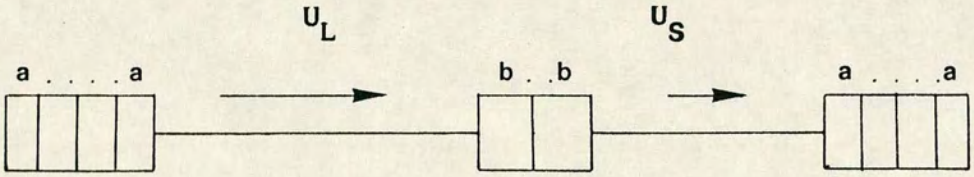

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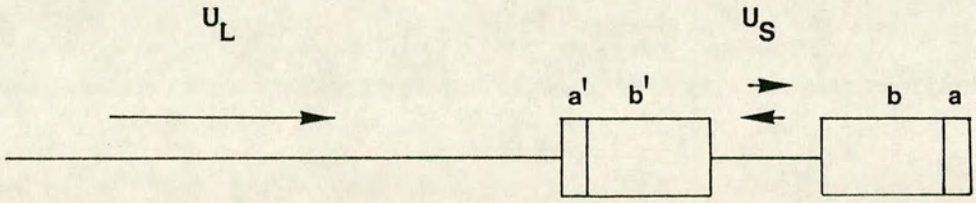
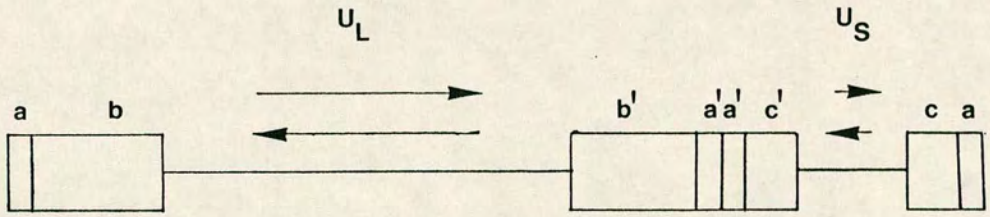


E



F

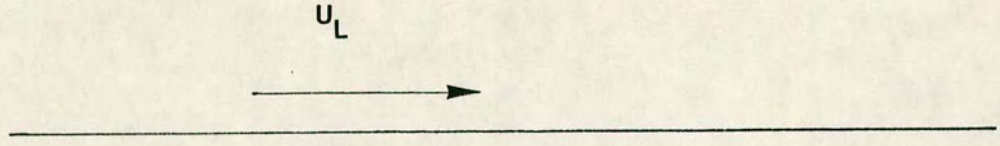


Figure 1.2.2.1
Classification of the herpesviruses according to their genomic organisation.

A **Example**: Channel catfish virus (CCV)
In CCV, terminal sequences make up 12% of the genome.

B **Examples**: *Herpesvirus saimiri* (HVS); *Herpesvirus ateles* (HVA)
The total number of repeat sequences is constant, but the number at each terminus varies between molecules. Termial sequences make up 30% of the genome in HVS.

C **Example**: Epstein Barr virus (EBV)

D **Examples**: *Equine herpesvirus* 1 (EHV-1); pseudorabies virus.

E **Examples**: *Herpes simplex virus* types 1 and 2 (HSV-1 and -2); human cytomegalovirus
Four isomers exist, therefore some 0.25 molar bands are observed on restiction endonuclease analysis.

F **Examples**: *Tupaia herpesvirus*; murine cytomegalovirus
No repeat sequences are present; the genomes are large (200 and 235 kbp respectively).

References:

A-E Roizman, 1982; Honess and Watson, 1977
F Koch *et al.*, 1985; Ebeling *et al.*, 1983

the characteristics of latent infection. Alpha herpesviruses undergo short reproductive cycles and often lie latent in ganglia. Beta herpesviruses undergo long reproductive cycles and lyse host cells slowly, often resulting in cytomegalia (cell enlargement) and cellular inclusions. Gamma herpesviruses infect lymphoid cells, and frequently remain latent within these cells. This classification is summarised in Table 1.2.1.1.

A further subdivision of gammaherpesviruses is often made into B-lymphotropic or gamma-1-herpesviruses, for example Epstein-Barr virus (EBV), and T-lymphotropic or gamma-2--herpesviruses, for example *Herpesvirus saimiri* (HVS) (Honess, 1984). The affinity of AHV-1 for T-lymphocytes in the species which it affects has resulted in its classification as a gamma-2-herpesvirus.

1.2.2 Classification of AHV-1 according to its genomic organisation.

Herpesviruses differ in the arrangement of repeated sequences in the genome. The different genomic arrangements are described in Figure 1.2.2.1. In general, alpha herpesviruses possess genome type D or E, while gamma-2 -herpesviruses possess genome type B. There is no consensus genomic organisation for beta herpesviruses.

1.3 The gamma-2-herpesviruses/ type B herpesviruses.

The term gamma-2-herpesvirus is a general term describing the T-lymphotropic properties of the herpesviruses concerned and it includes at least two herpesvirus genuses. The gammaherpesvirus genus *Rhadinovirus* comprises *Saimiriine herpesvirus* 2 (*H.saimiri* or HVS), *Ateline herpesvirus* 2 (*H.ateles* strain 810 or HVA) and *Ateline herpesvirus* 3 (*H.ateles* strain 73). The *Thetalymphocryptovirus* genus comprises *Gallid herpesvirus* 2 (Marek's disease virus) and *Meleagrid herpesvirus* 1 (Turkey herpesvirus) (Brown, 1986).

Other herpesviruses provisionally classified as gamma-2-herpesviruses by Honess (1984) were *Leporid herpesvirus* (*H.sylvilagus*), *Aotine herpesvirus* 2 (*H.aotus* type 2) and *Alcelaphine herpesvirus* 1. The bovine cytomegaloviruses, which have been classified as betaherpesviruses on the basis of their biological properties (Osorio and Reed, 1983), were also included because they have been shown to possess a type B genomic organisation (Storz *et al.*, 1984 and Ehlers *et al.*, 1985).

All of these viruses with the exception of the related Marek's disease virus and turkey herpesvirus possess a type B genomic organisation (Figure 1.3.2). The latter two viruses possesses a genomic organisation similar to those of the alpha herpesviruses (Buckmaster *et al.*, 1988) and therefore will not be discussed further in this thesis.

The gamma-2-herpesviruses with a type B genome, which will be designated *H.saimiri*-like herpesviruses in this thesis, are thus HVS, HVA, *H.aotus* type 2 and *H.sylvilagus*. The biology of HVS, HVA and *H.aotus* type 2 are similar. These viruses induce no disease in their naural hosts, the squirrel monkey, spider monkey or owl monkey respectively, but cause lymphomas in other New World monkeys (Falk, 1980b and Fuchs *et al.*, 1985). *H. sylvilagus* differs from these viruses in being capable of inducing disease in its natural host, the cotton-tail rabbit (Hinze 1971).

The affinity of AHV-1 for T-lymphocytes and the presence of tandem repeat sequences (see Section 1.4) suggest that *Alcelaphine herpesvirus* 1 (AHV-1) is similar to the *H.saimiri*-like herpesviruses, and therefore the genomic characteristics of this group will be discussed.

1.3.1 The genomic organisation of *H.saimiri*-like herpesviruses.

Genomes of the *H.saimiri*-like herpesviruses comprise a linear double-stranded DNA molecule of 110-170 kbp. The genome lengths have been estimated by electron microscopy,

the summation of restriction endonuclease fragments, or sedimentation coefficients (for example, Cohrs and Rouhandeh, 1987).

The *H.saimiri*-like herpesviruses all possess a distinctive heterogeneity in guanine and cytosine (G+C) content in different regions of the genome. The heterogeneity of the viral genomes of HVS and HVA with respect to G+C content was first indicated by their biphasic DNA melting profiles and by the DNA peaks produced by isopyknic centrifugation of these DNAs in caesium chloride density gradients before and after mechanical shear. When HVS DNA was analysed in the absence of shear, DNA banded at two densities corresponding to 70.6% and 45.4% G+C, with 10% of the DNA banding at the higher density. After shearing, the DNA banded at densities corresponding to 70.6% and 35.8% G+C. This implied that the 45.4% G+C DNA was sheared to both 70.6% and 35.8% G+C, and is explicable if two types of genome occur: -M genomes comprising light DNA (L-DNA), together with denser sequences designated heavy DNA (H-DNA) -H genomes of H-DNA only. (Fleckenstein *et al.*, 1975). Figures for the G+C content of HVS, HVA and *H.aotus* type 2 are shown in Table 1.3.1.1.

The location of the H-DNA within the M-genomes of HVS was determined by partial denaturation mapping followed by computer alignment of the denaturation maps (Bornkamm *et al.*, 1976). The HVS genomes denatured largely in the centre of the genome, while the termini remained as a duplex, which suggested a terminal location for the H-DNA. No fold-back structures were seen following this denaturation and reannealing, implying that inverted repeat sequences were not a feature of H-DNA.

Restriction endonuclease analysis and DNA hybridistion experiments have been used to confirm and extend these results. Digestion of the viral DNA wih an endonuclease which cuts within the repeated DNA (H-DNA) produces

	mole percentage guanine plus cytosine		
	unique DNA	repeat DNA	whole genome
H. saimiri	35.8	70.6	45.4
H. ateles	37.5	74.6	47.1
H. aotus type 2	40.2	68.7	48.8

Table 1.3.1.1

Guanine plus cytosine content of three gamma-2-herpesvirus DNAs.

Taken from Fleckenstein and Mulder, 1980

	Genome size (kbp)	Repeat size (bp)	Repeat number	Reference(s)
H. saimiri	153-161	1444	30-34	1
H. ateles	106	1600		2
		1400		3
H. aotus type 2	151	2700), 2300) related		
H. sylvilagus	108-129	553	1-40	4,5
Bovine cytomegalovirus	138-150	2750), 2350), 1950) related	15	6

Table 1.3.1.2

Physical properties of the type B herpesvirus genomes.

References:

1 Stamminger *et al.*, 1987

2 Fleckenstein *et al.*, 1978

3 Fuchs *et al.*, 1985

4 Cohrs and Rouhandeh, 1987

5 Medveczky *et al.*, 1989a

6 Ehlers *et al.*, 1985

multimolar fragments, the sizes of which summate to the repeat sequence length. Digestion of the viral DNA with an endonuclease which cuts in the unique region of the genome (L-DNA) but not in the repeat DNA produces fragment ladders. Each DNA fragment in the ladder comprises the region of the unique DNA between the repeat sequences and the restriction site nearest to this DNA, with an integral number of repeat sequences. The difference in size betweeen adjacent fragments within the ladder corresponds to the size of the repeat sequence. Such fragment ladders are seen because the number of repeat sequences present at either one terminus varies continuously from one to the maximum repeat number, while the total number of repeat sequences present per molecule is a constant (Stamminger *et al.*, 1987).

At the beginning of this study the only gamma-2--herpesviruses to have been characterised were HVS and HVA. Restriction maps had been derived for both these viruses (Fleckenstein and Mulder, 1980) and the entire unique region of HVS had been cloned into bacteriophage and lambda vectors (Knust *et al.*, 1983b). A considerable amount of data on the genomic organisaton of all the gammaherpesviruses has been published in the interim. Table 1.3.1.2 summarises the data published on the repeat sequences of these viruses. The repeat sequences of HVS (Bankier *et al.*, 1985) and *H.sylvilagus* (Medveczky *et al.*, 1989a) have also been sequenced.

1.4 Genomic characterisation of AHV-1.

The only published results on the genomic characterisation of AHV-1 at the beginning of this study were the very limited restriction profiles of Ludwig (1983). The only detailed molecular characterisation performed at that time was that of Dr A.J.Herring, none of which had been published, although some results were reported at the 10th International Herpesvirus Workshop at Oxford in 1983. The extent of Dr. Herring's results are described below.

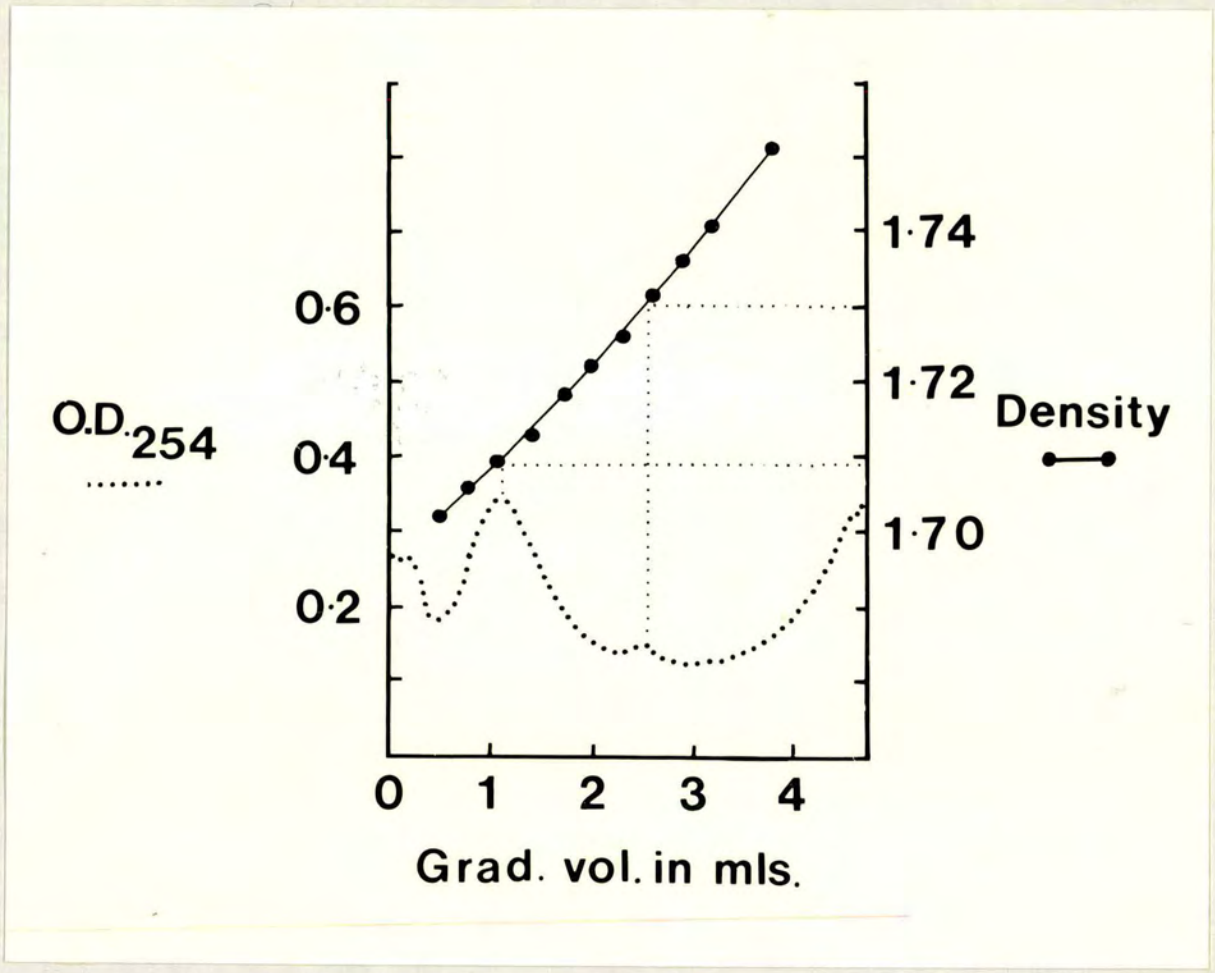


Figure 1.4.1
Caesium chloride density centrifugation of AHV1 DNA (WC11 isolate). 20 µg DNA was loaded on a 5ml 57% (w/v) gradient.

Early studies of AHV-1 included preparative caesium chloride density gradient centrifugation of viral DNA performed as the last stage of genome purification. The optical density profiles of the gradients revealed a major peak at a density of 1.709 and a minor but reproducible satellite component at a density of 1.730 g ml^{-1} (Figure 1.4.1). These densities correspond to G + C contents of 50% and 72% respectively (Schildkraut *et al.*, 1962). Such base sequence heterogeneity is typical of type B genome gammaherpesviruses.

AHV-1 DNA was restricted with a range of endonucleases including *Eco*RI, *Hin*dIII, *Hpa*II, *Mbo*I and *Sma*I. Of these enzymes, both *Hpa*II and *Sma*I produced a number of supermolar fragments, suggestive of the presence of repeated sequences. Both *Eco*RI and *Mbo*I produced ladders of fragments of equal molarity (discussed in Chapter 2).

A paper describing the genomic organisation of AHV-1 was produced during the course of this study (Bridgen *et al.*, 1989). This is included as Appendix 6.

1.5 Aims.

The general aim of this work was to characterise the genome of AHV-1. At the start of this study, there was almost no molecular characterisation of this virus, which is important both economically and for the information it may provide on the interaction of the components of the immune system. At the start of this work, there was also only indirect immunofluorescence antibody data to suggest that the SA-MCF agent was related to AHV-1, and it therefore seemed appropriate to apply a more sophisticated molecular genetic approach to this problem. The second biological problem to be assessed with the aid of AHV-1 viral clones was the study of the pathogenetic mechanisms of AHV-1. The results of these investigations are described in the next four chapters.

CHAPTER 2

ANALYSIS OF THE AHV-1 GENOME.

2.0 Introduction.

This chapter describes the characterisation of the AHV-1 genome. The majority of the experiments were performed using the attenuated WC11 isolate of AHV-1. Restriction analysis was used to compare DNA from this isolate with that from both cell-associated and cell-free forms of the virulent C500 isolate (C500 ca and cf, respectively). Viral clones were constructed from the WC11 isolate and used, together with restriction endonuclease data, to analyse the genomic organisation of AHV-1. The clones were also used to assess the homology of AHV-1 to other herpesviruses.

This work was always hindered by the difficulty of preparing sufficient quantities of AHV-1 DNA, particularly C500 DNA. This necessitated the use of sensitive DNA detection systems, including the silver-staining of DNA restriction fragments separated by polyacrylamide gel electrophoresis and DNA hybridisation. The non-radioactive labelling kit based on digoxygenin-dUTP produced by Boehringer became available towards the end of the work and this greatly facilitated the cross-hybridisation experiments required for the construction of a genomic restriction map for WC11 (discussed in Chapter 3).

The results described in this chapter and in Chapter 3 are discussed in Chapter 6.

2.1 Restriction data for AHV-1.

Restriction endonuclease analysis of WC11 and of C500 ca and cf DNA indicated that, in the unique DNA, C500 ca and cf were essentially identical, and that both differed only slightly from WC11 (see ahead to Fig. 2.2.1a and not illustrated). The repeated sequences of C500 ca and cf appeared to be identical, and they were much simpler than those of WC11 (discussed in section 2.2). The majority of the restriction endonuclease results were obtained for WC11 DNA and these results are discussed below.

Figures 2.1.1 and 2.1.2 show the profiles produced following restriction endonuclease digestion of WC11 DNA with a total of fifteen endonucleases. The figures are complementary, with Fig. 2.1.2 showing the fragments in the 0.5-4 kbp range for a number of digests seen in Fig. 2.1.1., in which larger fragments are resolved. Fragment mobilities vary slightly between the two gels; the discontinuously buffered PAGE system generally appeared to give slightly different mobilities from those obtained with both continuously buffered PAGE gels and agarose gels. However the former provided considerably greater fragment separation in the 1-4 kbp range than continuously buffered gels.

The enzymes *Hin*dII, *Sac*I and *Sma*I produced supermolar fragments which are clearly visible in Figs. 2.1.1 b) track 2 and c) track 7 and in 2.1.2 tracks 1 and 11. Supermolar fragments are indicative of the presence of repeated sequences. A total of ten enzymes was found to produce supermolar fragments and these are listed in Table 2.1.1, together with the estimated number of restriction sites in the WC11 genome for all the enzymes employed. The nature of the AHV-1 repeated sequences is described in Section 2.2.

An obvious difference between the profiles observed in Figs. 2.1.1 and 2.1.2 lies in the additional supermolar fragments of approximately 1.3 kbp observed in the *Eco*RI, *Kpn*I, *Sac*I and *Sal*I tracks of Fig. 2.1.1. Identical additional fragments were seen when C500 ca DNA was digested with these enzymes. These supermolar fragments showed no homology with the supermolar fragments discussed above. The fragments were only observed in DNA prepared from virus -infected cells, although a similar fragment appears in the *Eco*RI profile published by Rossiter *et al.* (1989). The DNA in that instance was prepared from virions which were not gradient purified. The significance of these fragments is unknown.

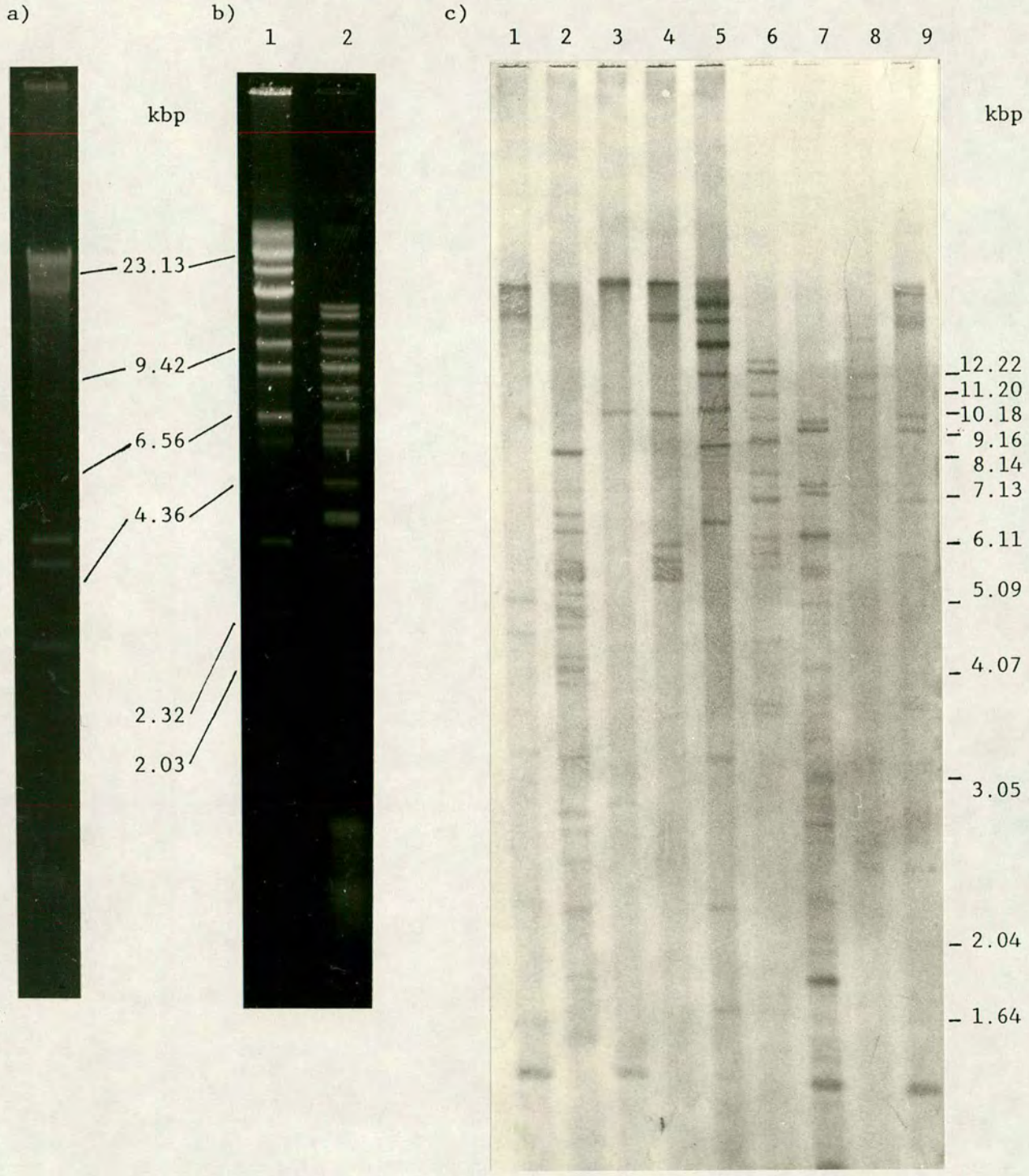


Figure 2.1.1
Agarose gel electrophoresis of WC11 DNA.

a) WC11 *Eco*RI (0.4% agarose)
b) Track 1: WC11 *Xho*I; track 2: WC11 *Sma*I (0.4% agarose)

c) WC11 DNA was separated through 0.9% agarose, transferred to nylon and the blot probed with digoxygenin-labelled WC11 DNA. The tracks show WC11 DNA digested as follows:

Track 1: *Eco*RI	4: *Bam*HI	7: *Sac*I
2: *Hin*dIII	5: *Xho*I	8: *Sac*II
3: *Sal*I	6: *Sma*I	9: *Kpn*I.

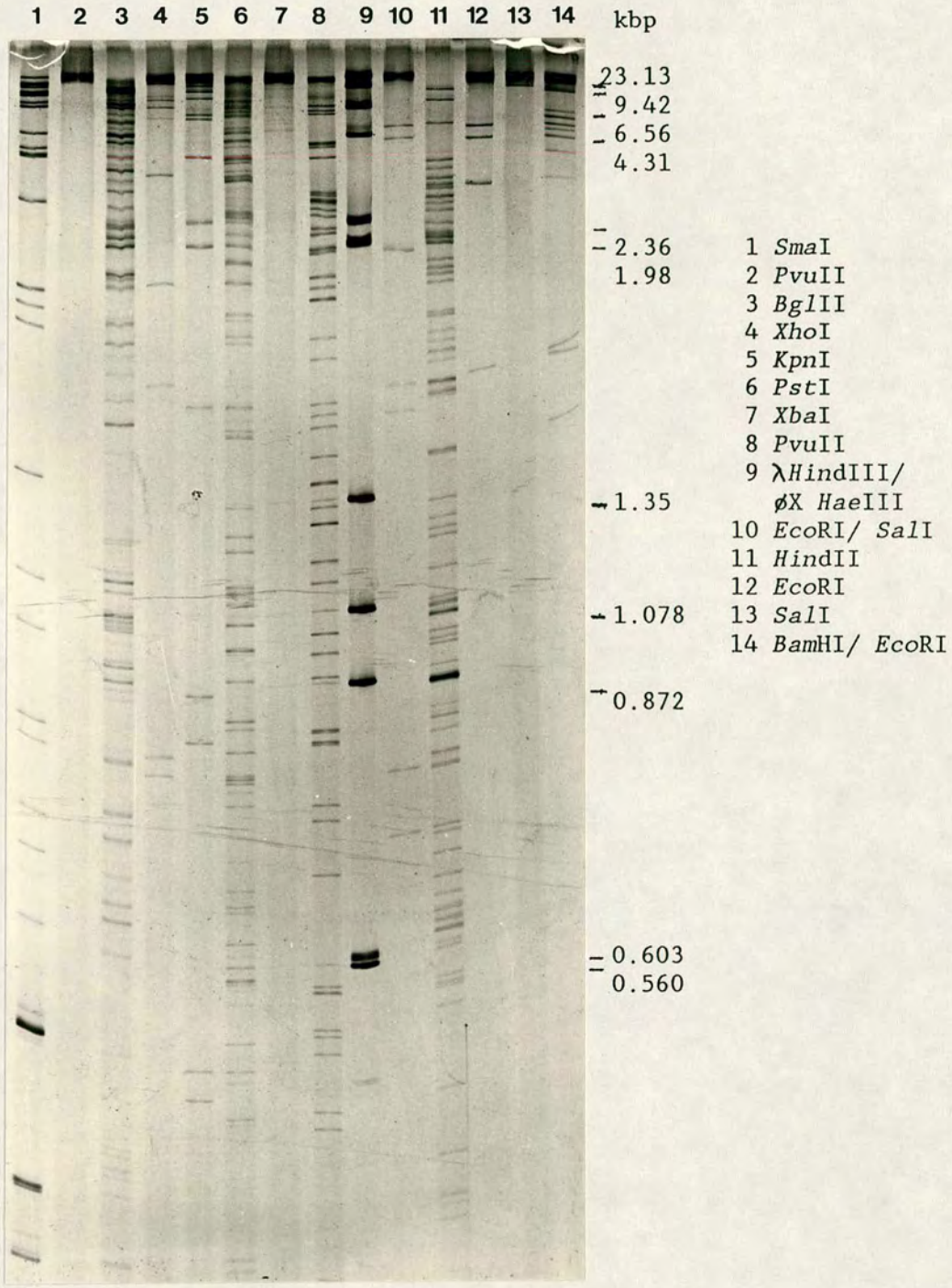


Figure 2.1.2
Siver-stained discontinuous polyacrylamide gel showing WC11 DNA digested with the restriction endonucleases indicated.

Table 2.1.1

The number of restriction sites for 23 restriction endonucleases in the unique and repetitive DNA of WC11.

ND = not determined.

Enzyme	Cleavage site	No. sites in unique DNA	No. sites in repeat DNA
*Alu*I	AG/CT	ND	2
*Apa*I	GGGCC/C	ND	⩾4
*Ava*I	G/G(A,T)CC	ND	6
*Bam*HI	G/GATCC	9	0
*Bgl*II	A/GATCT	>50	0
*Eco*RI	G/AATTC	8	0
*Hae*III	GG/CC	ND	⩾8
*Hha*I	GCG/C	ND	5
*Hin*dII	GTPy/PuAC	>60	1
*Hin*dIII	A/AGCTT	>20	0
*Hpa*II	C/CGG	ND	⩾6
*Kpn*I	GGTAC/C	12	0
*Not*I	GC/GGCCGC	ND	0
*Pst*I	CTGCA/G	>60	0
*Pvu*I	CGAT/CG	ND	0
*Pvu*II	CAG/CTG	>50	0
*Sac*I	GAGCT/C	>20	1
*Sac*II	CCGC/GG	ND	⩾1
*Sal*I	G/TCGAC	3	0
*Sau*3A	/GATC	ND	0
*Sma*I	CCC/GGG	>27	6
*Xba*I	T/CTAGA	ND	0
*Xho*I	C/TCGAG	15	0

2.2 The nature of the repeated sequences of AHV-1.

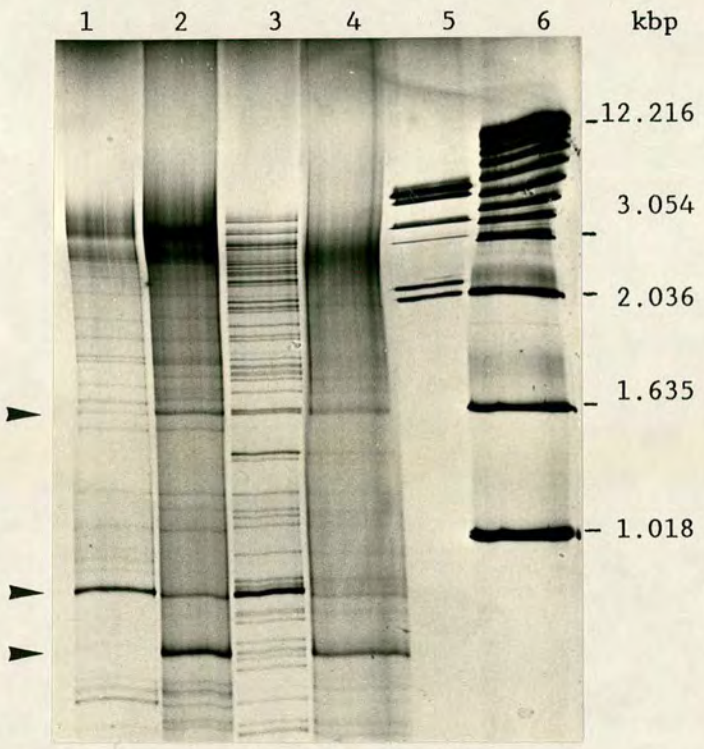
The existence of repeated sequences in AHV-1 was first suggested by the heterogeneous G+C content of the viral DNA revealed by caesium chloride density gradient centrifugation (Section 1.4). This is a distinctive feature of the gamma-2-herpesviruses, which possess repeated sequences with a high G+C content (Section 1.3.1). Restriction analysis confirmed that the satellite peak of high G+C AHV-1 DNA shown in Figure 1.4.1 produced *Mbo*I fragment ladders (Dr A.J. Herring, personal communication) and thus comprises repeated sequences (see below).

The presence of supermolar fragments confirmed the existence of repeated sequences, and indicated that they are arranged in a head-to-tail fashion. The supermolar fragments of C500 are much simpler than those of WC11. *Mbo*I, *Hin*dII and *Sac*I all produced a single supermolar fragment in C500 cf and ca DNA (Fig. 2.2.1a and not shown). In contrast, *Hin*dII and *Sac*I produced three supermolar fragments in WC11 DNA and these occurred at different levels of abundance, that is, at non-molar ratios. The supermolar fragments produced by *Hin*dII and *Sac*I digestion of WC11 DNA were identical. As discussed previously, discontinuously buffered gels maximise resolution in the 1-3 kbp range as compared with continuously buffered systems, but provide aberrant size estimates. Use of agarose gel electrophoresis and continuous PAGE indicated a size of 1050 bp for the single supermolar fragment of C500 cf , and 1800, 1050 and 950 bp for the three WC11 supermolar fragments.

Figure 2.2.1 b, c and d shows densitometric scans of Fig. 2.2.1a tracks 1, 2 and 3, respectively. The peak areas corresponding to unique and supermolar fragments have been compared to give an estimate of 20-25 copies of the single *Hin*dII/*Sac*I C500 repeat per genome. Estimates for the WC11 repeat sequence copy numbers are 4-5, 4-5 and 15-20 for the 1800, 1050 and 950 bp sequences respectively (see Appendix 2). The 950 bp *Hin*dII repeat sequence of WC11 was

Figure 2.2.1.

a) 1 C500 cf *Sac*I
2 WC11 *Sac*I
3 C500 cf *Hin*dII
4 WC11 *Hin*dII
5 λcI857 *Hin*dIII
6 1 kbp ladder



a) Silver-stained polyacrylamide gel showing the supermolar fragments produced by *Hin*dII and *Sac*I digestion of WC11 DNA. The supermolar fragments are arrowed.

b), c), d) Densitometric scan of Fig. 2.2.1a)

b) C500 cf *Sac*I. Peaks 1 and 2 correspond to unique fragments, peak 3 to the 1050 bp repeat sequence.

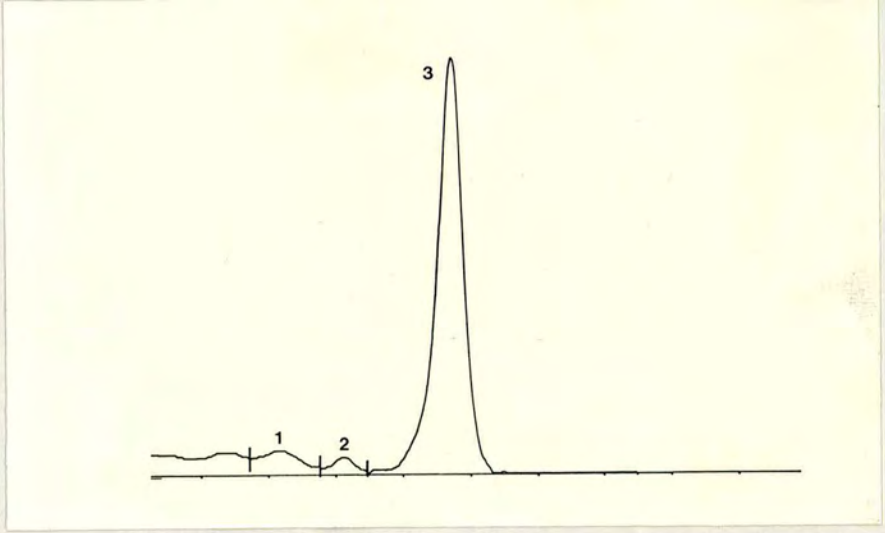
c) WC11 *Sac*I. Peaks 2, 3 and 4 correspond to unique fragments. Peaks 1, 5 and 6 correspond to the 1800, 1050 and 950 bp repeat sequences respectively.

d) C500 cf *Hin*dII. Peaks 1, 2, 4 and 5 correspond to unique fragments, peak 3 to the 1050 bp repeat sequence.

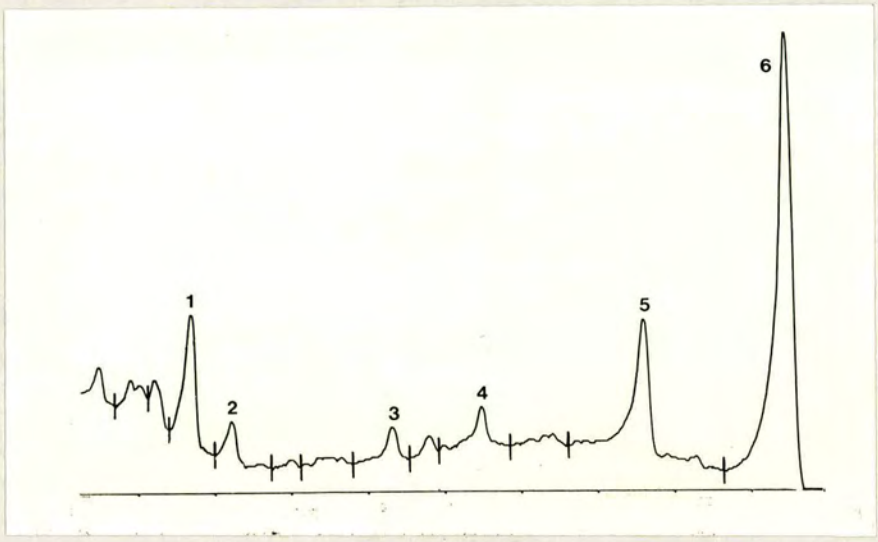
e) Densitometric scan of an autoradiogram showing hybridisation of whole ^{32}P-labelled virion DNA to a Southern blot of *Eco*RI-digested DNA. The large peaks correspond to hybridisation of unique DNA to the 5.2 and 4.8 kbp *Eco*RI fragments. The many smaller peaks represent hybridisation of the labelled repeated sequences to the *Eco*RI fragment "ladder". At least 10 peaks can be resolved.

The x and y axes of the densitometric scans correspond to distance and absorbance, respectively.

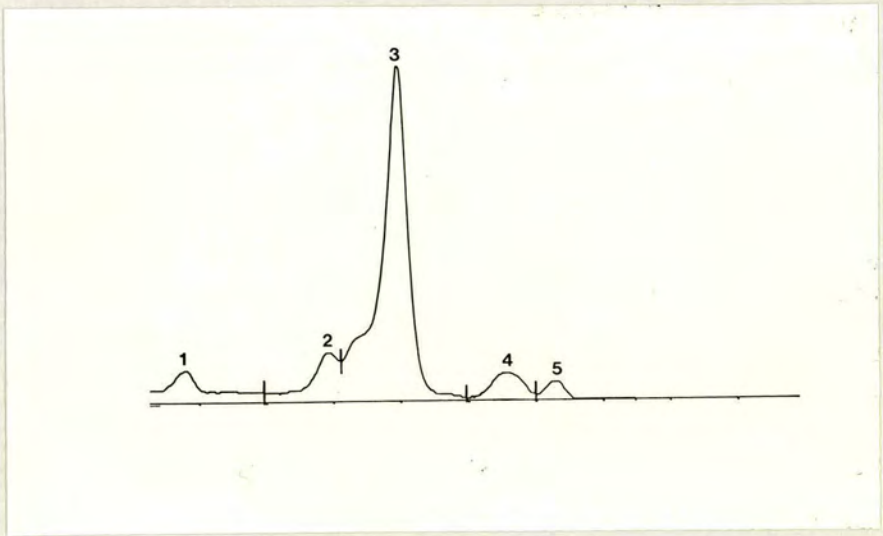
b)



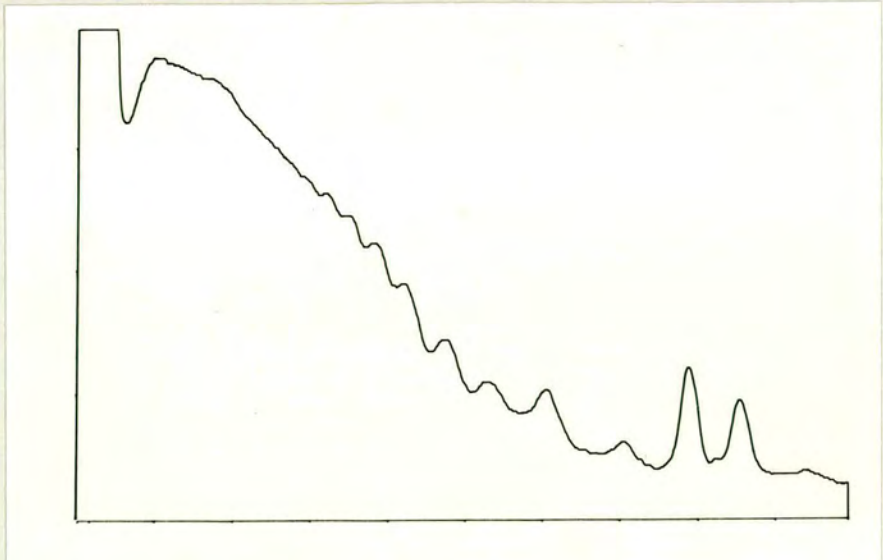
c)



d)



e)



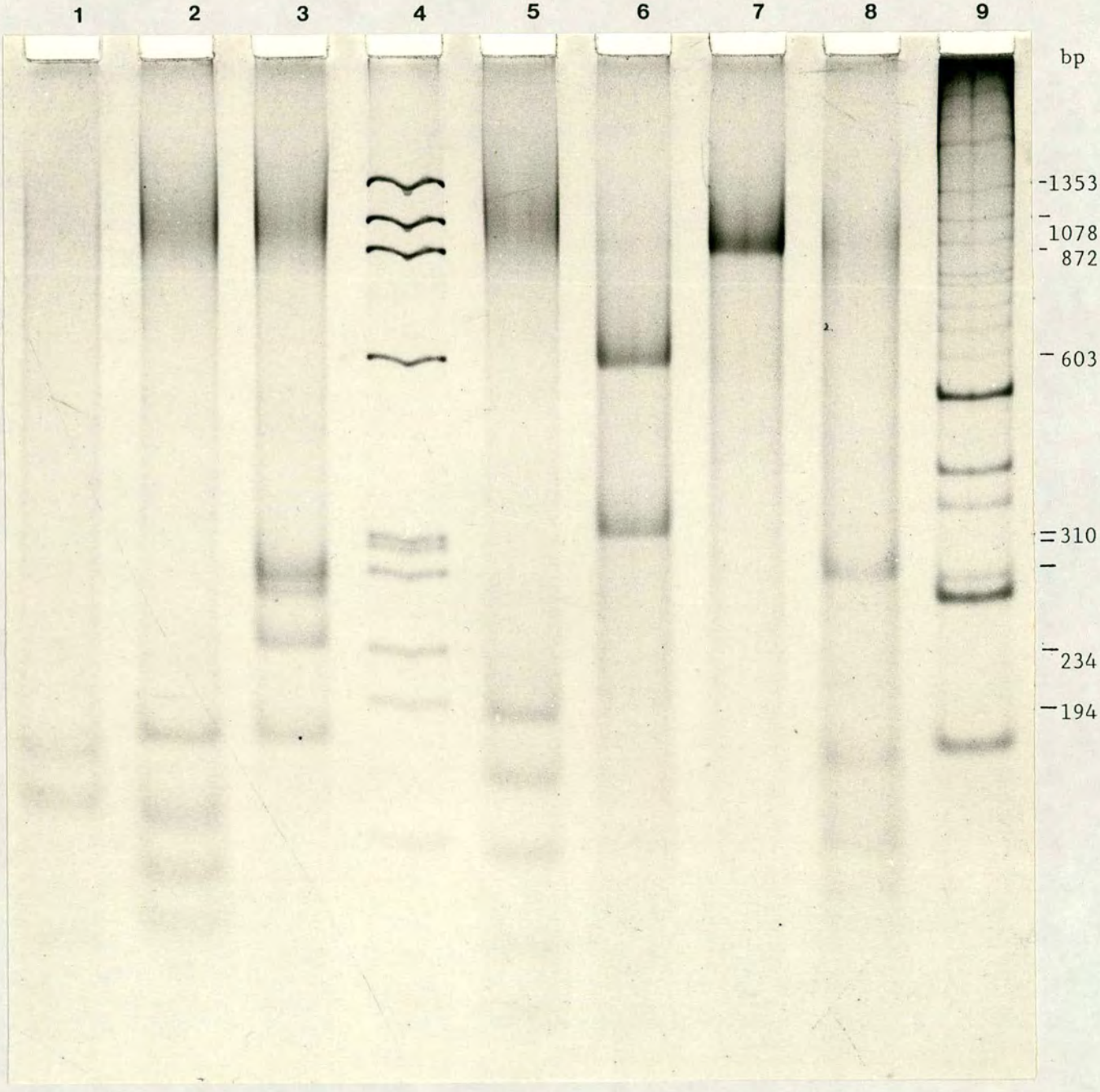
cloned into M13mp18 (clone M30, see Section 2.3.4); hybridisation experiments using this clone showed that all the supermolar fragments of C500 and WC11 produced by *Hin*dII, *Sac*I or *Sma*I digestion hybridised with the cloned sequence.

The differences in abundance of the WC11 repeated sequences can also be seen in the *Sma*I profile of Fig. 2.2.2, track 9, which shows three major *Sma*I repeats with mobilities corresponding to 165, 275 and 525 bp and four minor *Sma*I fragments with mobilities of 285, 360 and 430 bp. In contrast, track 3 shows *Sma*I digestion of the 950 bp *Hin*dII repeated sequence isolated from an agarose gel by the freeze squeeze method of Thuring *et al.* (1975). There are fragments of 165, 230, 275 and 295 bp with those of 230 and 295 bp corresponding to the digested 525 bp fragment. All these fragments occurred in molar ratios. Direct *Sma*I analysis of C500 cf DNA showed the 1050 bp repeat to consist of fragments of 165, 275, 285 and 360 bp (data not shown). The additional 285 and 360 bp fragments appear as minor fragments in the WC11 repeats, suggesting that the C500 and WC11 *Hin*dII and *Sac*I 1050 bp fragments are equivalent. The 1800 bp WC11 *Hin*dII fragment was shown to contain a 430 bp *Sma*I fragment (not illustrated) thus accounting for all the minor WC11 *Sma*I fragments. The differences between the repeated sequences of WC11 and C500 could result from initial strain variation or from modification of the WC11 genome during culture.

Evidence that the repeated sequences reside in the genome as head-to-tail arrays in two blocks of variable length is provided by the following observations. Restriction endonuclease profiles for enzymes which do not produce supermolar fragments all show large fragments of indistinct resolution and/or fragment ladders with a periodicity equivalent to the major repeat size of 950 bp. Both these structures showed homology to the repeat sequence clone M30. For example, *Eco*RI digests showed a single ladder of

Figure 2.2.2
Siver-stained continuous PAGE showing the WC11 950 bp major *Hin*dII repeat sequence isolated from an agarose gel digested as follows:

1 *Hae*III
2 *Ava*I
3 *Sma*I
4 øX 174 *Hae*III markers
5 *Hpa*II
6 *Alu*I
7 *Sau*3A
8 *Apa*I
9 *Sma*I-digested WC11 DNA



fragments which hybridised to M30 ranging in size from 5 to greater than 20 kbp, while a second ladder of 8 to greater than 20 kbp was revealed when the *Eco*RI digest was probed with the unique region clone H48, which is known to be terminally located (see section 3.1). Fragment ladders were also observed when *Bam*HI, *Eco*RI, *Hin*dIII, *Mbo*I and *Xho*I -digested WC11 DNA was probed with the repeat sequence clone M30. In the cases of *Hin*dIII (not shown) and *Mbo*I (Fig. 2.2.3a) two distinct ladders were observed.

The explanation for the fragment ladders is as follows. The ladders were only observed following digestion with restriction endonucleases which did not cut in the repeated sequences. A terminal location for the repeated sequences coupled with continuously variable numbers of repeated sequences on each terminus of a given molecule would, on restriction digestion, result in the production of two sets of fragments containing repeated sequences. Each series would comprise the unique DNA located between the restriction site nearest to the repeats and the repeated sequences, together with an integral and continuously variable number of these repeated sequences. The autoradiogram produced following hybridisation of M30 to *Eco*RI-digested WC11 DNA (Fig. 2.2.1e) resolves more than ten ladder components while that produced following hybridisation to *Mbo*I-digested WC11 DNA resolves at least twelve ladder components. Thus, there must be at least twelve repeated sequences, and probably somewhat more than this. This is consistent with the results derived from densitometric scanning of size-fractionated *Hin*dII or *Sac*I-digested WC11 DNA (Fig. 2.2.1 b, c and d; see Appendix 2).

Closer analysis of the *Mbo*I ladders seen in Fig. 2.2.3a and b shows that the resolution of the ladder decreases with increasing molecular weight; this is seen most clearly in the overloaded track (4) of Fig. 2.2.3b. This is thought to result from repeat sequence variability, which could possibly occur as a result of internal deletion.

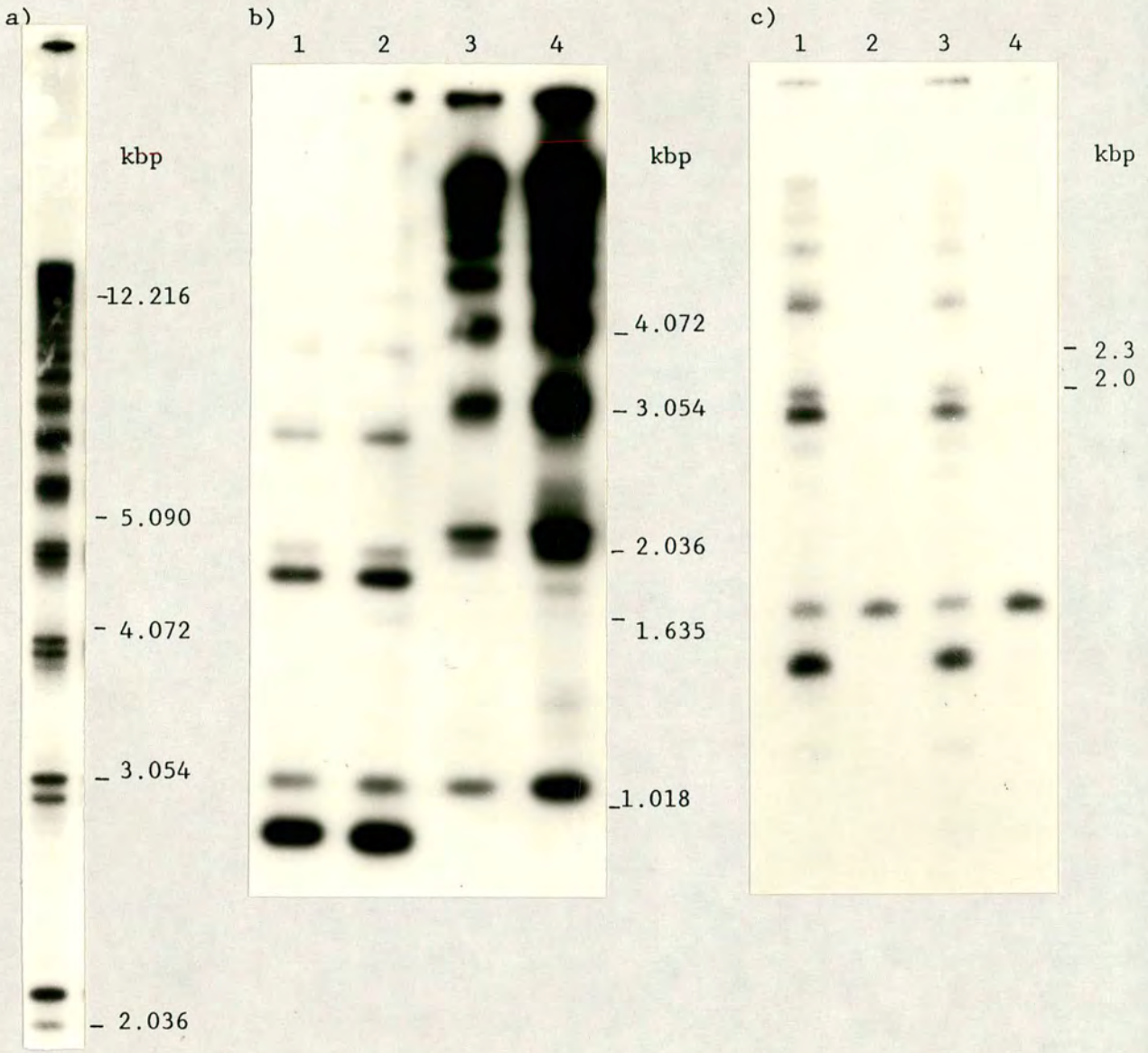


Figure 2.2.3
Autoradiograms showing fragment "ladders":

a) Southern blot of WC11 infected-cell DNA digested with *Mbo*I, separated through 1% agarose and probed with M30, the 950 bp major *Hin*dII repeat sequence clone.

b) Southern blot of WC11 infected-cell DNA digested with *Sac*I (track 1), *Hin*dII (track 2) or *Mbo*I (tracks 3 and 4) separated through 1.2% agarose and probed with M30.

c) Southern blot of WC11 and C500 cf DNA digested with *Hin*dII or *Sac*I, separated through 2% agarose and probed with M30.
Track 1: WC11 *Hin*dII
2: C500 cf *Hin*dII
3: WC11 *Sac*I
4: C500 cf *Sac*I

Interspersion of the 950 bp sequences with, for example, smaller variants of the 950 bp sequence or 1050 bp sequences which have lost their *Mbo*I site, would result in a complex ladder similar to that illustrated.

The complexity of the repeats is confirmed by Fig. 2.2.3b and c, which show the autoradiograms produced following hybridisation of M30 to *Hin*dII and to *Sac*I-digested WC11 and C500 DNA. Single hybridising fragments are seen following hybridisation of M30 to *Hin*dII and *Sac*I-digested C500 DNA, but the fragments seen on hybridisation to digested WC11 DNA also form a ladder. The hybridisation signal strengths decrease with increasing molecular weight, in an identical fashion for the two digests, suggesting that the larger fragments occur at a lower abundance. This would tend to confirm the hypothesis of repeat sequence variation involving loss of the *Hin*dII or *Sac*I sites. That the 1800 bp *Hin*dII/*Sac*I repeat sequence revealed by hybridisation with M30 forms a part of the *Hin*dII/*Sac*I ladder of fragments shown in Fig. 2.2.1 suggests that the 1800 bp repeat sequence may in fact comprise a variant 950 bp repeat sequence dimer. It is conceivable that the 950 bp sequence itself comprises a combination of several smaller sequences, which would explain the tendency of the sequence to delete. This would also explain why restriction digestion of the 950 bp major *Hin*dII repeat with, for example, *Hpa*II, produced a number of small fragments which summated to a value less than 950 bp.

The terminal location of the repeated sequences has not been proved directly. Digestion of WC11 DNA with the progressive exonuclease *Bal*31 failed to remove all the repeat DNA. However, the WC11 restriction map is consistent with a terminal location for the repeats (see Section 3.5).

2.3 Molecular cloning of WC11 DNA.

The construction of the WC11 clones is described in this chapter, while mapping of these clones is described in

Chapter 3. The vectors used for the majority of the cloning work were the lambda vectors λEMBL3, λEMBL4 and λNM1149. These were chosen for their large insert capacity (9-22 and 0-9 kbp for the λEMBL and λNM1149 vectors respectively) and the ease of selection of recombinant molecules on the appropriate host strain. Plasmid pUC13 was employed for subcloning, and the sequencing vector M13mp18 for the cloning of the WC11 950 bp repeat sequence. The initial cloning experiments involved cloning of *Eco*RI fragments into λEMBL4, the attempted cloning of *Bam*HI fragments into λEMBL3 and the cloning of *Hin*dIII fragments into λNM1149. The cloning of *Eco*RI fragments into λNM1149, the subcloning of some fragments and the construction of the repeat clone took place at a later date.

2.3.1 Construction of the λEMBL4-*Eco*RI clones.

λEMBL4-*Eco*RI recombinants of AHV-1 were constructed by ligation of *Eco*RI-digested WC11 DNA with *Eco*RI/*Bst*I -digested λEMBL4 DNA. The recombinant molecules were packaged and plated on 5K cells, and recombinant molecules determined by their *spi*$^-$ phenotype and hybridisation to DNA extracted from WC11-infected BET cells. Large-scale DNA preparations were made from twelve of the eighty-six recombinants. Five of these clones were found to possess multiple small *Eco*RI inserts, for example R53 contained inserts of 1.5, 3.4 and 4.8 kbp. Two clones, designated R54 and R65, were found to possess a 19 kbp insert. Restriction analysis indicated that the inserts were identical, but were cloned in opposite orientations.

2.3.2 Construction of the λNM1149-*Hin*dIII clones.

λNM1149-*Hin*dIII recombinants of AHV-1 were constructed by ligation of *Hin*dIII-digested WC11 DNA to *Hin*dIII-digested λNM1149 DNA. The recombinant molecules were packaged and plated on NM514 cells. 127 recombinants were obtained. Small-scale DNA preparations from each phage were made as described (Section 7.7.6) or DNA was extracted with phenol from phage grown on agarose plates. The small-scale

preparations gave unreliable results, therefore large-scale DNA preparations were made for 21 of these clones. Restriction endonuclease analysis and cross hybridisations were performed to analyse and distinguish the clones.

2.3.3 Construction of the λNM1149-*Eco*RI clones.

WC11 DNA digested with *Eco*RI was ligated to *Eco*RI -digested vector DNA, and recombinant molecules recovered by transfection of NM514 cells. Approximately 150 plaques were obtained which showed positive hybridisation to WC11 DNA. Small-scale DNA preparations were made from 20 of these recombinants, and the insert sizes determined. The clones designated X1, X3, X5 and X12 possessed inserts of 5.2, 1.5, 4.8 and both 4.8 and 3.4 kbp respectively. The validity of these clones was assessed by hybridisation of the labelled clones to a blot of *Eco*RI-digested WC11 DNA. All four clones hybridised to WC11 *Eco*RI fragments which co-migrated with the clone insert(s). The 4.8 kbp inserts of X5 and X12 were found to cross-hybridise. Homology was also detected with some of the λNM1149-*Hin*dIII clones which possessed *Eco*RI sites; X1 hybridised to H44, X5 hybridised to H6, and X12 hybridised to H6 and H48 (see Section 3.1). Restriction profiles of the clones were derived by single and double digestions of the phage DNA and by comparison with the λNM1149-*Hin*dIII clone restriction maps.

2.3.4 Construction of the M13mp18-*Hin*dII repeat clone.

The major *Hin*dII repeated sequence of WC11 was cloned to aid the analysis of the location and organisation of the AHV-1 repeated sequences and for use in *in situ* hybridisation experiments. M13mp18 DNA was digested with *Sma*I, which possesses a unique restriction site within the multiple cloning region. The WC11 major *Hin*dII repeat sequence of 950 bp was isolated from a 0.5% agarose gel by the method of Thuring *et al.* (1975), and ligated to the linearised vector. Recombinant molecules were recovered by transfection of NM522 cells and were assessed for homology

to AHV-1 DNA by hybridisation with the labelled 950 bp *Hin*dII repeat fragment.

Small-scale RF DNA preparations were made from 10 of these recombinants. The DNA was digested with *Sma*I, separated by agarose gel electrophoresis, blotted and the filters were probed again with the 950 bp *Hin*dII repeat sequence. Four of the recombinants analysed showed the 275 and 175 bp *Sma*I fragments typical of the WC11 repeated DNA. One of these, designated M30, was chosen for subsequent experimental work. The validity of this clone was confirmed by restriction of WC11 and M30 DNA with *Sma*I and with *Ava*I; both DNAs produced fragments which co-migrated during continuous PAGE.

2.4 Homology of AHV-1 to other herpesviruses.

The AHV-1 unique region clone R65 (19 kbp insert size) and the repeat sequence clone M30 (950 bp insert size) were used to assess the homology of AHV-1 DNA to the DNA of three other gammaherpesviruses (EBV, HVS and *H.sylvilagus*), to *Bovid herpesvirus* 4 (BHV-4, V/test strain) and to *Human herpesvirus* 6 (HHV-6). The gammaherpesvirus DNAs comprised the cloned *Bam*HI-W fragment of EBV, HVS genomic DNA and unique and repeat sequence clones of *Herpesvirus sylvilagus*. The homology to BHV-4 DNA was assessed because of reports of immunological reactivity of AHV-1 to this virus (Rossiter *et al.*, 1988) and because of the similarity of the genomic organisation of this herpesvirus to the gammaherpesviruses (Storz *et al.*, 1984 and Ehlers *et al.*, 1985). HHV-6, like AHV-1, is a highly cell-associated lymphotropic herpesvirus. ~~but (it was later discovered) it possesses an alphaherpesvirus-like genomic organisation~~. Genomic DNA from the alphaherpesvirus BHV-1 (infectious bovine rhinotracheitis virus) was used as a negative hybridisation control in some experiments because of the extremely high (71%) G+C content (Goodheart and Plummer, 1975).

M30, labelled with [α^{32}P]-dATP by nick tanslation, hybridised to a 2.6 kbp *Eco*RI fragment of BHV-4 DNA under conditions of low stringency (the hybridisation was performed at 42°C in hybridisation solution containing 30% formamide and 4 x SSC). *Eco*RI-digestion produces three fragments of this size in BHV-4 DNA, one of which is the high G+C repeat sequence; it is likely that this represented non-specific hybridisation to this repeat DNA. M30 did not hybridise to any of the DNAs at medium stringency (50% formamide, 4 x SSC, 42°C).

R65 labelled by random primer extension hybridised to an 11.25 kbp *Msp*I fragment (*Msp*I-C) of HVS DNA (Fig. 2.4.1). Under conditions of low stringency R65 showed much stronger hybridisation to HVS DNA than to an equal loading of BHV-1 DNA, which possesses a considerably higher G+C content in the unique DNA. The non-specific hybridisation to BHV-1 disappeared at medium stringency, while hybridisation was still observed to the HVS *Msp*I-C fragment. Hybridisation of R65 to *Eco*RI, *Xho*I and *Hin*dIII digests of HVS DNA localised the region of homology to a 2.3 kbp *Hin*dIII fragment contained within *Eco*RI-C, located just 5' to the 2.7 kbp immediate-early transcript (Bodemer *et al.*, 1984, Stamminger *et al.*, 1987). This region is known to contain 500 bp of a simple repeat, which could be the cause of the cross -hybridisation (Dr R.W. Honess, personal communication).

The region of the R65 clone which hybridised to HVS DNA was determined by hybridisation of digoxygenin-labelled R65 subclones (Section 3.1) to blots of *Msp*I-digested HVS DNA under low stringency hybridisation conditions (30% formamide, 4 x SSC and 42°C; the filters were washed in 2 x SSC at 50°C for two periods of 15 minutes). No hybridisation was detected to HVS DNA using the sublones H10 or p6f but subclone p6a showed medium hybridisation to HVS *Msp*I-C and weak hybridisation to HVS *Msp*I-A.

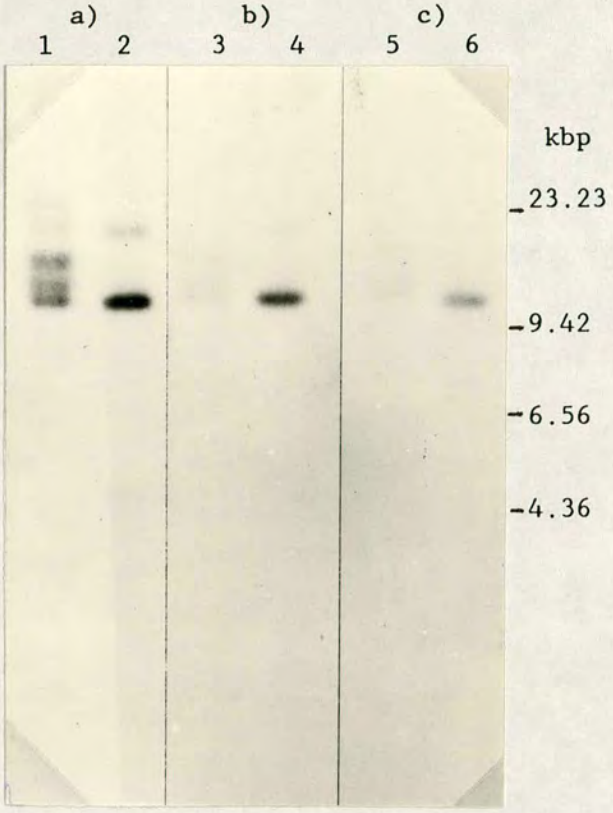


Figure 2.4.1

Autoradiogram showing hybridisation of the WC11 clone R65 labelled with [α^{32}P]-dATP to *Hin*dIII-digested BHV-1 DNA (tracks 1, 3 and 5) and *Msp*I-digested HVS DNA (tracks 2, 4 and 6) at different stringencies. Hybridisations were performed at 42°C overnight in hybridisation solution containing 4 x SSC and a) 20% formamide b) 30% formamide and c) 40% formamide. Hybridisation to HVS DNA was still detectable following hybridisation at 42°C in hybridisation solution containing 4 x SSC and 50% formamide (not shown).

Recent preliminary sequence analysis of a small part of the p6a DNA (subcloned as a 1.5 kbp *Sau*3A fragment in M13mp18) revealed that the following sequence was repeated at least 2-3 times:

-GGGGACCCCGAGGGACCAGATGGAACTGAAGGAGAAGGCCCATGCGGACCAGGTGGGCC
AGATGAAGAC-

It is possible, therefore, that this cross-hybridisation result betweeen AHV-1 and HVS DNA is not of great significance, since both viral genomes possess repeated DNA in or near the region of sequence similarity. Comparison of the small sequenced region of the p6a M13 subclone with sequences available in the EMBL databank indicated a 60% overlap in 67 nucleotides with ORF 69 of the alphaherpesvirus *Varicella zoster* virus (VZV). The p6a insert may therefore correspond to a viral region conserved between numerous herpesviruses.

WC11 genomic DNA and clones R65 and M30 were also used to probe DNA from a virus submitted by a collaborating laboratory. The circumstances of the isolation of this virus suggested that it might be the agent of SA-MCF. The M30 clone hybridised to fragments produced by *Sac*I-digestion of this DNA identical and unique to those of WC11 DNA. This parallels the situation described in Section 1.1.2.2, where the putative SA-MCF agent isolated by Hambdy *et al.* (1978) was found to be an AHV-1 contaminant.

CHAPTER 3

DERIVATION OF THE WC11 RESTRICTION MAP.

3.0 Introduction.

This chapter, which builds on the results described in Chapter 2, describes the experiments which allowed the derivation of a restriction map for WC11 DNA with respect to *Bam*HI, *Eco*RI, *Sal*I, *Sma*I and *Xho*I.

The derivation of a restriction map for the WC11 genome with respect to *Eco*RI was chosen as a suitable starting point for a more complete restriction map since *Eco*RI is one of the few endonucleases known to produce a moderate number of fragments. Seven fragments are produced of 45, 35-40, 19, 5.2, 4.8, 3.4 and 1.5 kbp, respectively, in addition to two fragment ladders. The five smallest fragments were cloned in lambda vectors (see Section 2.3). Thus R65 comprises the cloned 19 kbp fragment; X1, X5 and X3 comprise the cloned 5.2, 4.8 and 1.5 kbp fragments, respectively, while X12 comprises both the 4.8 and 3.4 kbp fragments. The sizes of the two largest *Eco*RI fragments were determined using the restriction map for WC11 discussed in this chapter, although the map obtained did not provide accurate sizing of the 35-40 kbp fragment (see Section 3.2.7). λNM1149-*Hin*dIII clones covered 60% of the 45 and 35-40 kbp *Eco*RI fragments. The *Eco*RI map, restriction analysis of all the cloned fragments and numerous cross-hybridisation experiments between these clones and different digests of WC11 DNA were employed in the construction of a consistent viral restriction map for *Bam*HI, *Sal*I and all but the smallest *Sma*I and *Xho*I fragments.

3.1 Derivation of the WC11 *Eco*RI map.

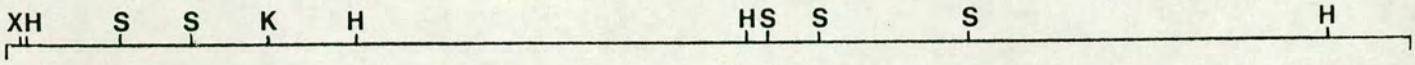
The WC11 *Eco*RI map was derived using the clones described in Fig. 3.1.1. The λNM1149-*Eco*RI clones X1, X3, X5 and X12 and the λNM1149-*Hin*dIII clones H1, H6, H10, H44 and H48 were mapped with respect to *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, *Sma*I and *Xho*I by single and double restriction digestion. R65 proved hard to map by these means, therefore pUC13 *Hin*dIII subclones were constructed and mapped individually and the

Figure 3.1.1

The WC11 clones used in the construction of the *Eco*RI map.
B=*Bam*HI, H=*Hin*dIII, K= *Kpn*I, R=*Eco*RI, S=*Sma*I, Sa=*Sal*I and X=*Xho*I.
1cm represents 1 kbp. Only R65 was mapped with respect to *Kpn*I.

a) λEMBL4 clone, *Eco*RI insert

R65 19 kbp insert

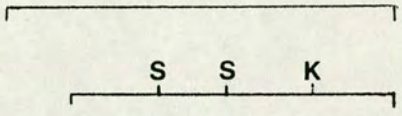


b) pUC13 clones, *Hin*dIII inserts

p4e 8.0 kbp insert (identical to that of H10)

p6a 5.2 kbp insert

p6f 4.4 kbp insert



c) λNM1149 clones, *Hin*dIII inserts

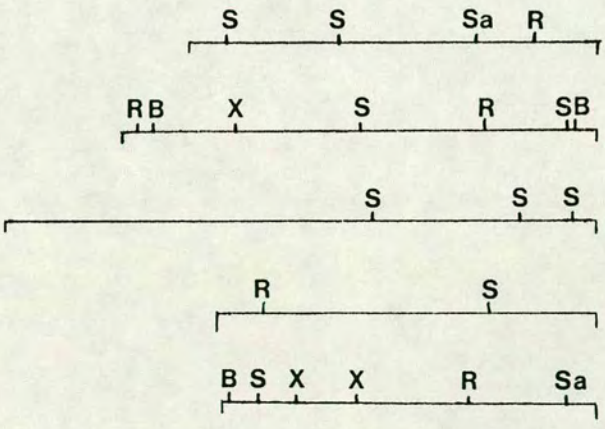
H1 5.5 kbp insert

H6 6.4 kbp insert

H10 8.0 kbp insert

H44 5.1 kbp insert

H48 5.0 kbp insert



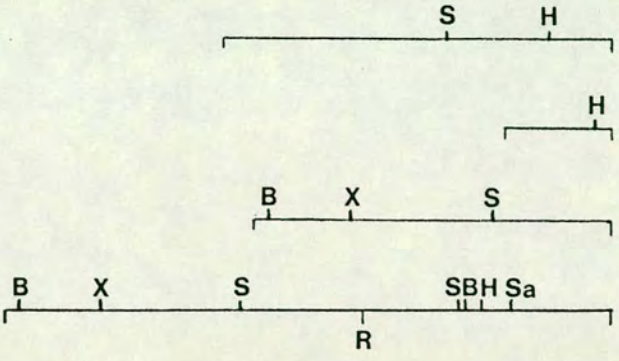
d) λNM1149 clones, *Eco*RI inserts

X1 5.2 kbp insert

X3 1.5 kbp insert

X5 4.8 kbp insert

X12 4.8, 3.4 kbp inserts



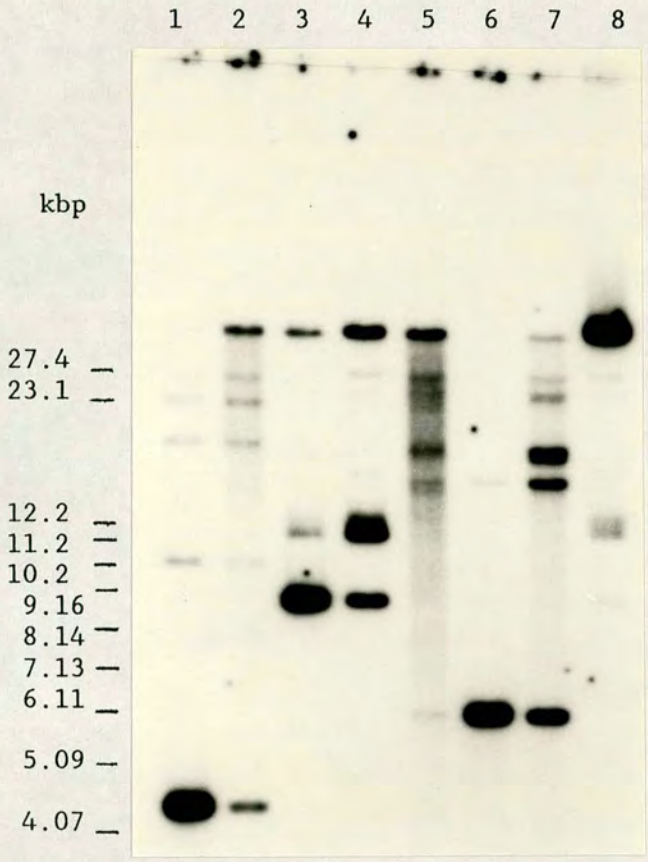


Figure 3.1.2

Autoradiogram showing partially digested and 3′ cos-end labelled R65 and H10 DNA. Each sample contains digested material pooled from reactions stopped at 2, 6 and 20 minutes.

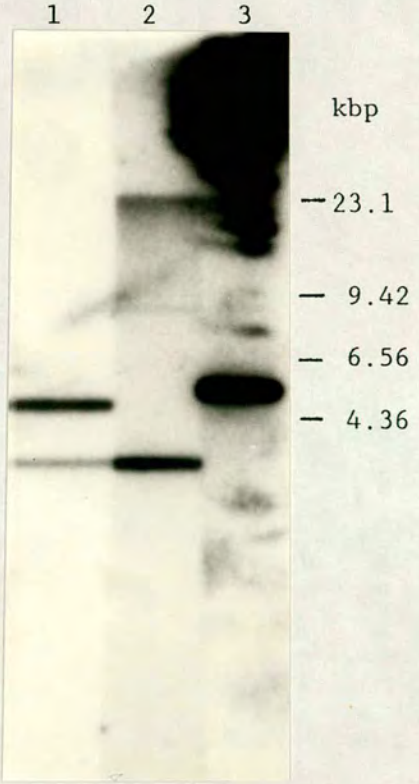


Figure 3.1.3

Autoradiogram showing hybridisation of the ^{32}P -labelled clones:

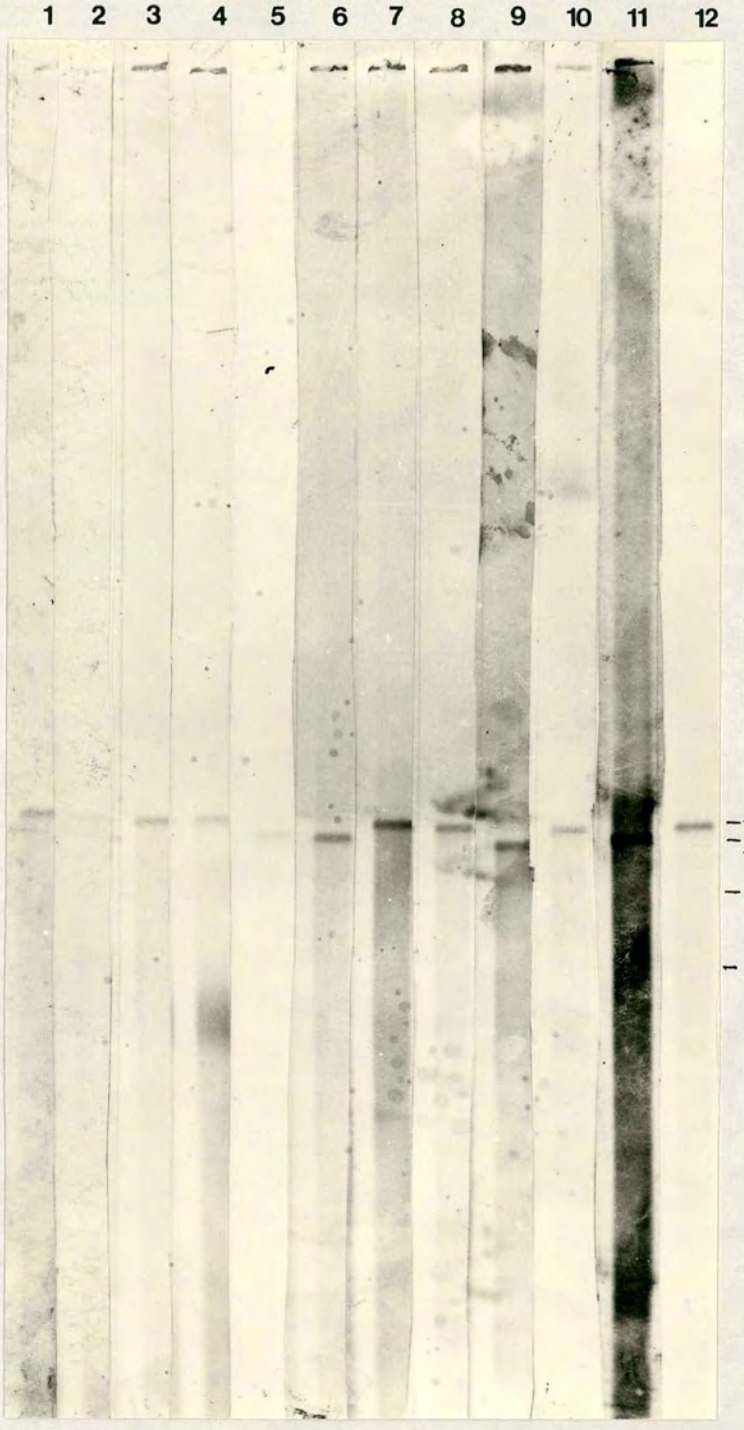
1 H6
2 H48
3 H44

to a Southern blot of *Eco*RI -digested WC11 DNA. Note the ladder of fragments present in track 2.

order of the subclones determined by restriction analysis or by partial digestion and cos-end labelling. p4e, p6a and p6f are different *Hin*dIII-pUC13 subclones of R65, with inserts of 8.0, 5.2 and 4.4 kbp respectively. Their inserts are equivalent to those of the NM1149-*Hin*dIII clones H10, H26 and H21a/H22, where H21a indicates the larger of the two H21 inserts. The position of subclone p6f within R65 was determined by the position of its *Kpn*I site. The small *Hin*dIII/*Eco*RI fragments present at the 5′ and 3′ ends of the R65 insert are of 370 and 940 bp, respectively; *Xho*I digestion splits the 370 bp *Eco*RI/*Hin*dIII fragment into a 220 bp *Hin*dIII/*Xho*I fragment and a 150 bp *Xho*I/*Eco*RI fragment (not illustrated).

The remaining two R65 subclones were ordered and orientated by partial restriction and cos-end labelling. Fig. 3.1.2 shows the autoradiogram produced during one partial digestion and cos-end labelling experiment. This method, based on Rackwitz *et al.* (1984), was used to map H10 with respect to *Sma*I, and R65 with respect to *Hin*dIII and *Sma*I. The fragment size estimations derived from Fig. 3.1.2 are shown in Appendix 3. The results from this experiment and from complete digestion of R65 and its subclones were combined to produce the clone map seen in Fig. 3.1.1a.

Several of the WC11 *Eco*RI fragments could be ordered by hybridisation of WC11 *Hin*dIII clones containing internal *Eco*RI sites to viral *Eco*RI blots. Fig. 3.1.3 shows hybridisation of labelled clones H6, H44 and H48 to a blot of *Eco*RI-digested WC11 DNA. H6 hybridised to the 4.8 and 3.4 kbp fragments while H44 hybridised to the 5.2 kbp WC11 *Eco*RI fragment. H48 hybridised to both the 3.4 kbp fragment and also to a ladder of fragments of between 8 and greater than 20 kbp. H1 did not hybridise to any of the five smallest *Eco*RI fragments (not shown). The ladder of fragments is likely to represent a terminal variable *Eco*RI fragment (see Section 2.2). These results indicated that



1 H36
2 H2
3 H9
4 H21
5 H1
6 H44
7 H17
8 H43
9 H39
10 H25
11 H37
12 H1 1 kbp internal *Eco*RI fragment

Figure 3.1.4

The figure shows a Southern blot of *Eco*RI-digested WC11-infected cell DNA separated through 0.3% agarose and probed with the digoxygenin-labelled WC11 clone probes as indicated.

the 4.8 kbp WC11 *Eco*RI fragment is adjacent to the 3.4 kbp fragment, which is iself adjacent to the variable 8 -20 kbp fragment. The clone H6 spanned the junction of the viral 4.8 and 3.4 *Eco*RI fragments while H48 spanned the junction between the viral 3.4 and variable 8-20 kbp fragments. This overlap is illustrated in Fig. 3.2.2 section e) at the end of the chapter.

The WC11 45 and 35-40 kbp *Eco*RI fragments (*Eco*RI-A and -B respectively according to the convention of designating viral fragments A-Z in order of decreasing size) could be separated by electrophoresis through 0.3% (w/v) agarose (see Fig. 3.1.4). H44 hybridised to *Eco*RI-B while H1 hybridised to both fragments. Hybridisation using H1 as the probe could only be detected to *Eco*RI-A following labelling of the entire clone, but was detected to *Eco*RI-B when the 1.0 kbp *Eco*RI fragment spanning the 3' vector-insert junction of H1 was used as the probe. These results indicated that the 5.2, 35 and 45 kbp *Eco*RI fragments of WC11 are adjacent in the genome in this order, with H44 spanning the 5.2-35 kbp *Eco*RI fragment junction and H1 spanning the 35-45 kbp *Eco*RI fragment junction. This is illustrated in Fig. 3.2.2 sections b) and c).

In order to determine the genomic locations of the WC11 *Eco*RI 1.5 and 19 kbp fragments a number of the clones were labelled and used to probe blots of *Sma*I and *Xho*I -digested WC11 DNA. H6 and X3 were both found to hybridise to a 12 kbp *Sma*I fragment and a 10 kbp *Xho*I fragment (see ahead to Table 3.2.2). These clones must therefore map to a similar position on the genome; since the larger *Hin*dIII-*Eco*RI fragment of X3 does not correspond to an internal *Hin*dIII-*Eco*RI fragment of H6 (see Figure 3.1.1) these clones must therefore overlap by a 200 bp *Hin*dIII/*Eco*RI fragment. X1 and H10 both hybridised to a 24 kbp *Xho*I fragment and an 8.2 kbp *Sma*I fragment (Table 3.2.2) therefore it was concluded that these clones contain fragments which map to the same region of the genome.

At this point the following WC11 *Eco*RI fragments are known to be adjacent:

1) The 19 (R65), 5.2 (X1), 35-40 and 45 kbp fragments and
2) The 1.5 (X3), 4.8 (X5/12), 3.4 (X12) and 8-20 kbp fragments

Since R65 did not hybridise to the *Sma*I and *Xho*I fragments homologous to X3 (Table 3.2.2), it was concluded that the 1.5 kbp *Eco*RI fragment must be adjacent to *Eco*RI-A (45 kbp). The unique WC11 *Eco*RI fragments must therefore be ordered:

- 19 - 5.2 - 35/40 - 45 - 1.5 - 4.8 - 3.4 -

(see Section 3.2.5 for a discussion of terminal fragments).

Figure 3.1.4 also reveals that one subclone of R65, H21, did not hybridise to a unique 19 kbp *Eco*RI fragment of WC11 DNA, but to a series of fragments of 19-21 kbp. The smaller insert of this clone also hybridised to *Eco*RI-A (discussed in Section 3.2.3). Similar results were obtained when the C500 isolate was digested with *Eco*RI and probed with R65 (not shown). Hybridisation of the R65 *Hin*dIII subclones p6a, p6f and H10, labelled with digoxygenin-dUTP, to a blot of *Hin*dIII-digested WC11 DNA showed that p6a and p6f hybridised to discrete *Hin*dIII fragments while H10 hybridised to a series of fragments of 8-10 kbp (not shown). This suggested that there was variability between viral molecules in the H10 region of the AHV-1 genome.

3.2 Derivation of the *Bam*HI, *Sal*I, *Sma*I and *Xho*I map for WC11.

The known restriction data for digestion of WC11 DNA with the endonucleases *Bam*HI, *Sal*I, *Sma*I and *Xho*I (Table 3.2.1) and restriction and hybridisation data for the clones (Fig. 3.1.1 and Table 3.2.2) were combined to construct a restriction map for all the *Bam*HI and *Sal*I fragments and for the largest *Sma*I and *Xho*I fragments of WC11 DNA. Since most of the clones were constructed using *Hin*dIII a partial *Hin*dIII map could also be constructed for WC11.

Table 3.2.1
Restriction fragment size estimations for WC11 DNA.

	Size (kbp)				
Fragment	*Bam*HI	*Eco*RI	*Sal*I	*Sma*I	*Xho*I
A	41	45	58-63var?	13.6	24
B	21	35/40	43	12.8	19
C	20	19-21	10	11.1	16
D	10	var	var	9.7	16
E	5.8	5.2		8.7	12
F	5.6	var		8.7	10
G	5.3	4.8		7.5	8.5
H	5.1	3.5		6.8	6.0
I	var	1.5		5.9	var
J	var			5.6	var
K				5.4	3.0
L				4.5	2.2
M	113.8	114.7		3.7	1.9
N	4.8	8.2		3.6	1.5
O	4.0	5.0		3.0	.82
P				1.75	.8
Q	122.6	127.9		1.7	
R				1.6	121.72
S				1.3	5.5
T				1.1	5.1
U				1.1	
V				.88	132.32
W				.85	
X				.76	
Y				.72	
Z				.64	
				123.0	

var = variable length fragment adjacent to repeats

The restriction fragments are summated both with and without the variable length fragments for the *Bam*HI, *Eco*RI and *Xho*I digests; *Sma*I produces no such fragments as it cuts within the repeat DNA.

The restriction fragment sizes below approximately 20 kbp were estimated following agarose gel electrophoresis, while those above this figure were estimated from the restriction map (see ahead to Fig. 3.2.2). For the summation of the *Eco*RI fragments the 19-21 kbp fragment was taken as 20 kbp while the 35/40 kbp fragment was taken as 35 kbp in the absence of conclusive evidence for a size estimation of 40 kbp (see Sections 3.0, 3.2.7).

Table 3.2.2
Hybridisation data for the WC11 clones (RI = *Eco*RI, H3 = *Hin*dIII).
The clones are listed in order of their genomic map position (5'-3').
ND=not determined; var=variable ladder fragment; ()=faint hybridisation.

Clone	Clone type	Insert size (kbp)	WC11 fragment(s) to which clone hybridises: *Eco*RI	*Bam*HI	*Sal*I	*Xho*I	*Sma*I
R65	λEMBL4 RI	19	19-21	40	ND	24	8.3,7.5,3.7, 2.0,0.9,0.7
p6f	pUC13 R65 H3 subclone	4.4	19-21	ND	ND	24	7.5,3.7,0.9
P6a	pUC13 R65 H3 subclone	5.2	19-21	ND	ND	ND	ND
H10	λNM1149 R65 H3 subclone	8.0	19-21	ND	ND	24	8.3,(7.5), 2.0,0.7
X1	λNM1149 RI	5.2	5.2	40	ND	24,1.6	13,8.2
H44	λNM1149 H3	5.1	35/40,5.2	ND	ND	24,1.6	13,8.2
H37	λNM1149 H3	8.0,1.5	35/40	40	ND	16	13,0.9
H2	λNM1149 H3	2.8 x 2	45,35/40	21,10	ND	19,16	8.3,5.8,5.2 1.6,0.5
H39	λNM1149 H3	4.3	35/40	21,10	ND	19	8.2
H1	λNM1149 H3	5.5	45,35/40	21	>40	19	5.6,1.3
H21b	λNM1149 H3	2.8	45	21	ND	19,16	(5.8),5.6
H43	λNM1149 H3	1.6	45	21	ND	16	5.8
H17	λNM1149 H3	6.2	45	21,5.4	ND	16	9.4,1.7,0.3
H32	λNM1149 H3	4.6,3.8 1.1	45	20,5.7 5.1	>40	16,13, 10,6.2	14,12,9.4 0.6
H9	λNM1149 H3	5.3	45	20,5.1	>40	13,6.2	14
H36	λNM1149 H3	3.8	45	20	>40	10	12
X3	λNM1149 RI	1.5	1.5	20	9.5	10	12
H6	λNM1149 H3	6.4	4.8,3.5, (1.5)	5.8	9.5	10,8.5	12,2.8
X5	λNM1149 RI	4.8	4.8	ND	ND	10,8.5	12,2.8
X12	λNM1149 RI	4.8,3.4	4.8,3.4	5.8,5.3	ND	10,8.5	12,4.4,2.8
H48	λNM1149 H3	5.0	3.4,var	5.3	9.5, var	8.5,0.8	5.4,4.4

The genomic map positions for all the WC11 clones were derived as follows. The clones were first used to probe a viral *Eco*RI blot in order to determine their approximate position; blots of *Bam*HI, *Sma*I and *Xho*I -digested viral DNA were then probed in order to position the clones more exactly. Hybridisation of clones to the 19, 5.2, 4.8, 3.4 and 1.5 kbp *Eco*RI fragments has been discussed (Section 3.1). Fig. 3.1.4 (page 52) shows hybridisation of the λNM1149-*Hin*dIII clones to the WC11 *Eco*RI-A and -B fragments. H9, H17 (=H25), H21, H36 and H43 hybridised to *Eco*RI-A (45 kbp); clones H37, H39 and H44 hybridised to *Eco*RI-B (35-40 kbp) and clones H1 and H2 hybridised to both (Fig. 3.1.4 and not shown). These results, restriction data for WC11 (Table 3.2.1) and restriction and hybridisation data for the clones were combined to construct the WC11 restriction map. Table 3.2.2 and Figure 3.2.2 (located at the end of this chapter) should be consulted throughout the following discussion.

3.2.1 The 5′ end of the WC11 restriction map.

The order of the *Hin*dIII clones which hybridised to R65 and X1 has been discussed (Section 3.1). Restriction and hybridisation data for these clones completely defines the restriction map for the region covering the 19 and 5.2 kbp WC11 *Eco*RI fragments (see Fig. 3.2.2 section a).

3.2.2 The WC11 restriction map covering *Eco*RI-B.

Four *Hin*dIII clones in addition to H44 mapped to WC11 *Eco*RI-B: H1, H37 and H39 and H2. H2 possesses two inserts of 2.8 kbp designated H2i and H2ii one of which, H2i, maps to this region. Of these clones, H37 hybridised to the same 13 kbp *Sma*I and 40 kbp *Bam*HI fragments as H44 and X1; it can therefore be concluded that H37 is located at the 5′ end of *Eco*RI-B. The 5′ end of the 13 kbp *Sma*I fragment is defined by clones H44 and X1; this information and the hybridisation of H37 to only one *Xho*I fragment of 16 kbp and only one *Sma*I fragment of 13 kbp define the extent of overlap between these two fragments, and the map position of H37, to within 1 kbp (Fig. 3.2.2 section b).

Hybridisation of H1, H2 and H39 to a viral *Bam*HI digest showed that these clones must map to the 3' end of the WC11 *Eco*RI-B fragment since none of these hybridised to the 40 kbp *Bam*HI fragment (see Fig. 3.2.2 sections b and c). H2 (which possesses no internal *Bam*HI site) and H39 (which possesses a single *Bam*HI site) both hybridised to 21 kbp and 10 kbp *Bam*HI fragments while H1 hybridised to a 21 kbp *Bam*HI fragment only. H39 must therefore span the junction of the 21 and 10 kbp *Bam*HI fragments, while H2 must have one insert within the 21 kbp and another within the 10 kbp *Bam*HI fragment. H1 spans the junction of *Eco*RI -A and -B and must therefore map to a position 3' to that of H39 and at least one insert of H2. Thus the AHV-1 *Bam*HI fragments must be ordered (5'-3'): - 40 - 10 - 21 - (Fig. 3.2.2 section c).

The exact map locations of clones H1, H2i (H2ii is discussed in more detail below) and H39 were determined by their restriction profiles in conjunction with the partial viral restriction map (see Fig. 3.2.2 section c). The position of H1 was determined by its *Eco*RI site (Fig. 3.1.1) and hybridisation properties to *Eco*RI-A and B (discussed in Section 3.1). The position of H39 with respect to the viral *Bam*HI fragments was determined by its *Bam*HI site (see ahead to Fig. 3.2.3) while hybridisation of H1 to *Bam*HI/*Eco*RI -digested WC11 determined the absolute position of the junction between the 21 and 10 kbp *Bam*HI fragments with respect to the viral *Eco*RI-A/B fragment junction. The position of H2i was then determined by its restriction profile for *Sma*I and the hybridisation data for the three clones to *Sma*I-digested WC11 DNA given in Table 3.2.1 (see Fig. 3.2.2. c).

The restriction and hybridisation data for clones H1, H2i and H39 completely define the WC11 restriction map at the 3' end of *Eco*RI-B (Fig. 3.2.2 section c). There is a slight region of uncertainty in the centre of *Eco*RI-B at the junction of the WC11 40 and 10 kbp *Bam*HI fragments, which

coincides with the junction of the *Xho*I 16 and 19 kbp fragments (Fig. 3.2.2 section b). This is discussed further in Section 3.2.7.

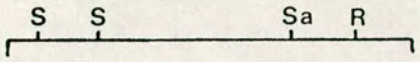
3.2.3 The WC11 restriction map covering *Eco*RI-A.

Six clones mapped to *Eco*RI-A (45 kbp): H2ii (=H21b) H9, H17, H32, H36 and H43 (see Fig.3.2.2 sections c, d and e for this discussion). H32 possesses three inserts, all of which map to this region, one of which, H32b, is identical to H36. Hybridisation of the clones to a *Bam*HI digest of WC11 DNA provided sufficient information to order three of these clones. Two clones possessed unique *Bam*HI sites and hybridised to two *Bam*HI fragments: H17 hybridised to *Bam*HI fragments of 21 and 5.8 kbp while H9 hybridised to *Bam*HI fragments of 20 and 5.1 kbp. H32 hybridised to *Bam*HI fragments of 20, 5.8 and 5.1 kbp, and possessed a single *Bam*HI site in the largest insert of 4.6 kbp. Since H1 hybridised to the 21 kbp *Bam*HI fragment, the *Bam*HI fragments spanned by H17 must be orientated as (5'-3') - 21 - 5.8 -. Since X3 (discussed in Section 3.1) hybridised to the 20 kbp *Bam*HI fragment, the *Bam*HI fragments spanned by H9 must be orientated (5'-3') - 5.1 - 20 -. The large insert of H32 must therefore span the junction of the 5.8 and 5.1 kbp *Bam*HI fragments and these results are consistent only with a *Bam*HI fragment order of - 21 - 5.8 - 5.1 - 20 -. This implies a clone order for H9, H17 and H32a of H17 - -H32a - H9. The exact map locations of these clones are discussed below.

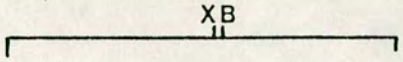
The orientation of the clones H9, H17 and H32a within the viral restriction map was determined as follows. Restriction profiles of these clones with respect to *Bam*HI, *Sma*I and *Xho*I are shown in Fig. 3.2.3. The *Sma*I sites and hybridisation data for H17 positioned this clone with respect to the WC11 *Sma*I fragments of 9.4, 1.7 and 0.3 kbp. H32a also hybridised to the WC11 9.4 kbp *Sma*I fragment and, since the *Sma*I site within this clone is at the vector -insert junction, the H32a clone must be immediately

Figure 3.2.3
Restriction data for selected WC11 clones.
B=*Bam*HI, H=*Hin*dIII, R=*Eco*RI, S=*Sma*I, Sa=*Sal*I and X=*Xho*I.
1cm represents 1 kbp.

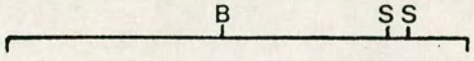
a) H1 5.5 kbp insert



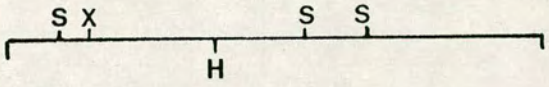
b) H9 5.3 kbp insert



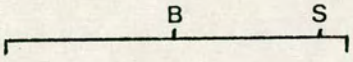
c) H17 6.2 kbp insert



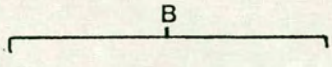
d) H21 inserts of 4.4 and 2.8 kbp



e) H32a insert of 4.6 kbp



f) H39 4.3 kbp insert



adjacent to H17 and orientated as illustrated for them both to fit within this *Sma*I fragment (Fig. 3.2.2 section c and d). The orientation of H32a determines the 5′ end of the viral 6.2 kbp *Xho*I fragment, and thus requires H9 to map immediately 3′ to H32a in order to fit with the restriction data obtained for H9.

The clones H21b/H2ii, H43, H36/H32b and H32c remain to be positioned within *Eco*RI-A. The hybridisation of H21b/H2ii to 5.6 and 5.8 kbp *Sma*I fragments, and the location of the *Sma*I site within this clone dictates a map position immediately 3′ to H1 for this clone. H43 also maps to the 5.8 kbp *Sma*I fragment, and is thus located 3′ to H21b/H2ii. H36 hybridised to the same *Bam*HI, *Sma*I and *Xho*I fragments as X3 (Table 3.2.2) and must therefore map to the 3′ end of *Eco*RI-A. This just leaves H32c; comparison of the hybridisation data for H36 and the entire H32 clone, specifically the hybridisation of H32 to a 12 kbp *Xho*I fragment, indicates that H32c maps to a position between that of H9 and H36. Isolation of this insert and hybridisation to blots of *Xho*I -digested WC11 DNA confirmed that H32c mapped to the WC11 12 kbp *Xho*I fragment. The clone order in *Eco*RI-A has thus been determined as:

- H21b/H2ii - H43 - H17 - H32a - H9 - H32c - H36 -

3.2.4 The 3′ end of the WC11 restriction map.

The 3′ region of the WC11 restriction map, in the region of the 1.5, 4.8 and 3.4 kbp *Eco*RI fragments is defined by restriction and hybridisation data for X12, X5, H6 and H48 discussed in Section 3.1 (see Fig. 3.2.2 section e).

3.2.5 Investigation of the unique-repeat DNA junction regions.

The sizes of the variable WC11 *Eco*RI fragments containing repeat sequences were estimated as follows. H48, which is located near the 3′ junction of the unique and repeated regions, hybridised to a ladder of fragments which varied in size from 8 to greater than 20 kbp (Fig. 3.1.3); the size of

the unique component of the DNA is therefore thought to be 7-8 kbp. H48 also hybridised to WC11 *Sma*I fragments of 4.4 and 5.4 kbp, of which the 5.4 kbp fragment is nearer the repeats (Fig. 3.2.2 section e). The position of the *Sma*I site in H48 indicated that the 3' *Sma*I site of the 5.4 kbp fragment mapped to a similar genomic position as the unique-repeat DNA junction of the 8-20 kbp variable *Eco*RI fragment; therefore the 5.4 kbp *Sma*I fragment may also span the unique-repeat DNA junction. Hybridisation of M30 to a *Sma*I fragment of 5 kbp (not illustrated) supports this idea.

Hybridisation of the repeat sequence clone M30 to a blot of *Eco*RI-digested WC11 DNA revealed a ladder of fragments varing in size from 5 to greater than 20 kbp. Since the 3' variable *Eco*RI fragment ladder has a minimum size of 8 kbp, the 5-8 kbp fragments must correspond to the smallest components of a second ladder and, therefore, the 5' unique-repeat junction *Eco*RI fragment must include 4-5 kbp of unique DNA.

3.2.6 Confirmatory results.

The results obtained in this Chapter were confirmed by the hybridisation of eight WC11 clones to double digests of WC11 DNA. The clones used were H1, H2, H9, H17, H36, H37 and H39, and these were used to probe some or all of blots of *Bam*HI/*Eco*RI, *Bam*HI/*Xho*I or *Eco*RI/*Xho*I -digested WC11 DNA. The results obtained were consistent with the derived map shown in Fig. 3.2.2.

3.2.7 Outstanding problems.

The junction between the 40 and 10 kbp *Bam*HI fragments, which coincides with the junction between the 16 and 19 kbp *Xho*I fragments is the least well defined region of the map. The 6.8 kbp *Sma*I fragment is almost certain to be located here, since the gap in the *Sma*I map is the only one large enough to accommodate this fragment. The data obtained during the course of this study were insufficient to prove that there were only four and not five *Bam*HI fragments of

between 5 and 6 kbp. There appear to be only four fragments visible in Fig. 2.1.1 track 4; therefore the map was constructed on this basis.

However, the following rather tenuous line of evidence suggests that there may, in fact, be five such fragments. Hybridisation of ^{32}P-labelled M30, the 950 bp repeat sequence clone of WC11, revealed a ladder of fragments of between 5 and greater than 20 kbp when used to probe *Xho*I-digested WC11 DNA. Were the the 3.0, 2.2, 1.9 and .82 kbp *Xho*I fragments, which have not yet been positioned on the WC11 map, to be located near the genomic termini, there could not be 5 kbp of unique DNA between the *Xho*I site and the junction of the unique and repeated DNA at both termini of the WC11 genome. Location of these *Xho*I fragments, and an additional *Bam*HI fragment within *Eco*RI-B, would resolve this problem. This would, of couse, extend the size of *Eco*RI-B to 40 kbp. However, the WC11 *Eco*RI and *Bam*HI fragments would then summate to approximately 130 kbp, a figure similar to that obtained for *Xho*I (Table 3.2.1). The *Sma*I fragments would still summate to only 125 kbp, but there are undoubtedly numerous very small *Sma*I fragments which could account for this difference.

Hybridisation of these *Xho*I fragments to blots of digested WC11 DNA would determine the map location of these fragments, but insufficient viral DNA has been available to do this. Further cloning of viral DNA will therefore be required to answer this question, and to determine the viral restriction map at the junctions between the unique and the repeated DNA. The 3′ end of the map is thought to be correct as shown, but the hybridisation of the clone H22 to a discrete rather than a variable WC11 40 kbp *Bam*HI fragment suggests that there is a *Bam*HI site between the 5′ repeated sequences and the start of the 19-21 kbp *Eco*RI site. Hybridisation of the R65 subclone p6f to a blot of *Kpn*I-*Bam*HI -digested WC11 DNA would resolve this point.

3.3 Size estimation of the WC11 genome.

Size estimation of the WC11 genome by electron microscopy proved difficult (see below) but a figure of approximately 160 kbp was provided by restriction analysis.

3.3.1 Size estimation of the WC11 genome by electron microscopy.

Appendix 4 shows the length measurements of 62 WC11 DNA molecules. The figures vary markedly, and it is likely that intrinsic nicks in the molecules coupled with breakages introduced on preparation of the DNA led to the production of many subgenomic DNA fragments.

The partial denaturation of some molecules was performed in an attempt to determine the location of the repeat sequences of WC11. In HVS DNA, conditions were achieved such that the unique DNA was denatured, but the G-C -rich repeat regions remained annealed (Bornkamm *et al.*, 1976). This was not achieved for AHV-1 DNA; no correlation was seen between denatured regions on different molecules.

3.3.2 Size estimation of the WC11 genome by restriction analysis.

The restriction map for WC11 DNA with respect to *Bam*HI, *Eco*RI, *Sal*I, *Sma*I and *Xho*I shown in Fig. 3.2.2 indicates that the unique region is 125 kbp in length; this could be a slight under-estimation as a result of lack of information on the the central portion of *Eco*RI-B (Section 3.2.7). 4-5 copies of both the 1050 and 1800 bp repeat sequences and 15-20 copies of the 950 bp repeat sequence lead to a estimate of 26-33 kbp for the repeat DNA size (Section 2.2). The overall genome size is thus estimated to be approximately 160 kbp.

3.4 Conclusion.

The results discussed in this chapter have proved sufficient to produce the restriction map for WC11 DNA shown in Fig. 3.2.2., and thus to estimate the length of the WC11 genome as approximately 160 kbp. The restriction map provides the first evidence for the terminal location of the repeated sequences of AHV-1.

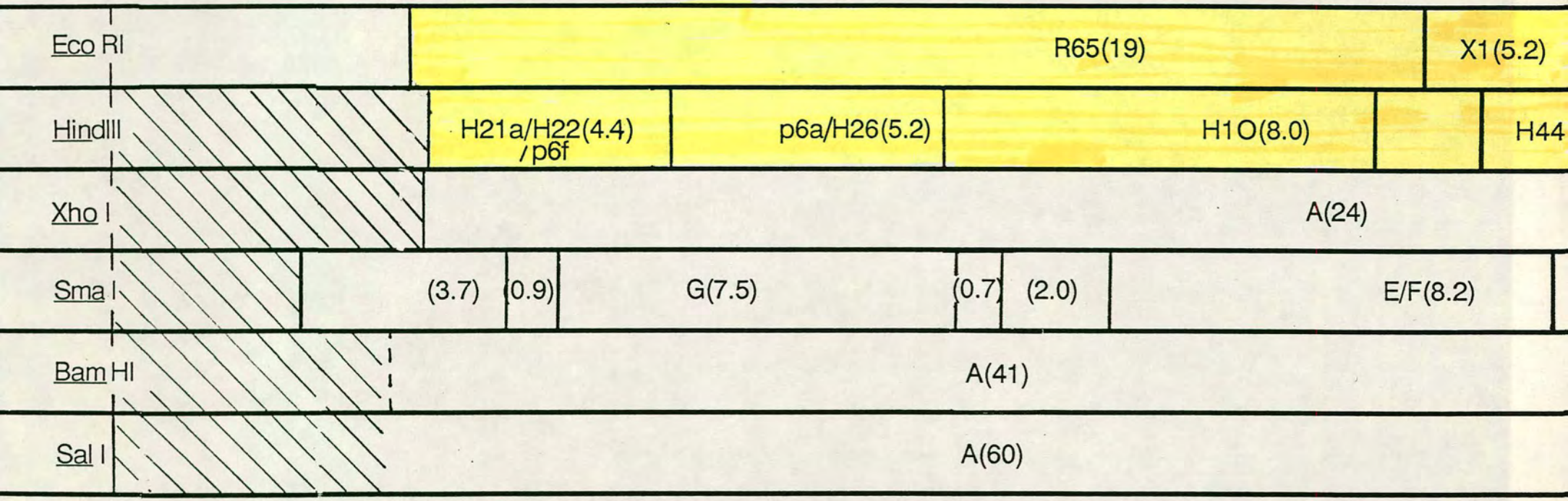
Figure 3.2.2 (following page)

The restriction map for the unique DNA region of the WC11 isolate of AHV-1 with respect to *Bam*HI, *Eco*RI, *Sal*I, *Sma*I and *Xho*I, showing the map positions of the unique region *Hin*dIII and *Eco*RI clones. 1 cm represents 1 kbp.

Cloned DNA regions are shaded; regions of the map which have not been determined for a particular endonuclease are hatched. Where WC11 clones cannot be mapped to a precise position on the genome, arrows indicate the region to which the clone must map.

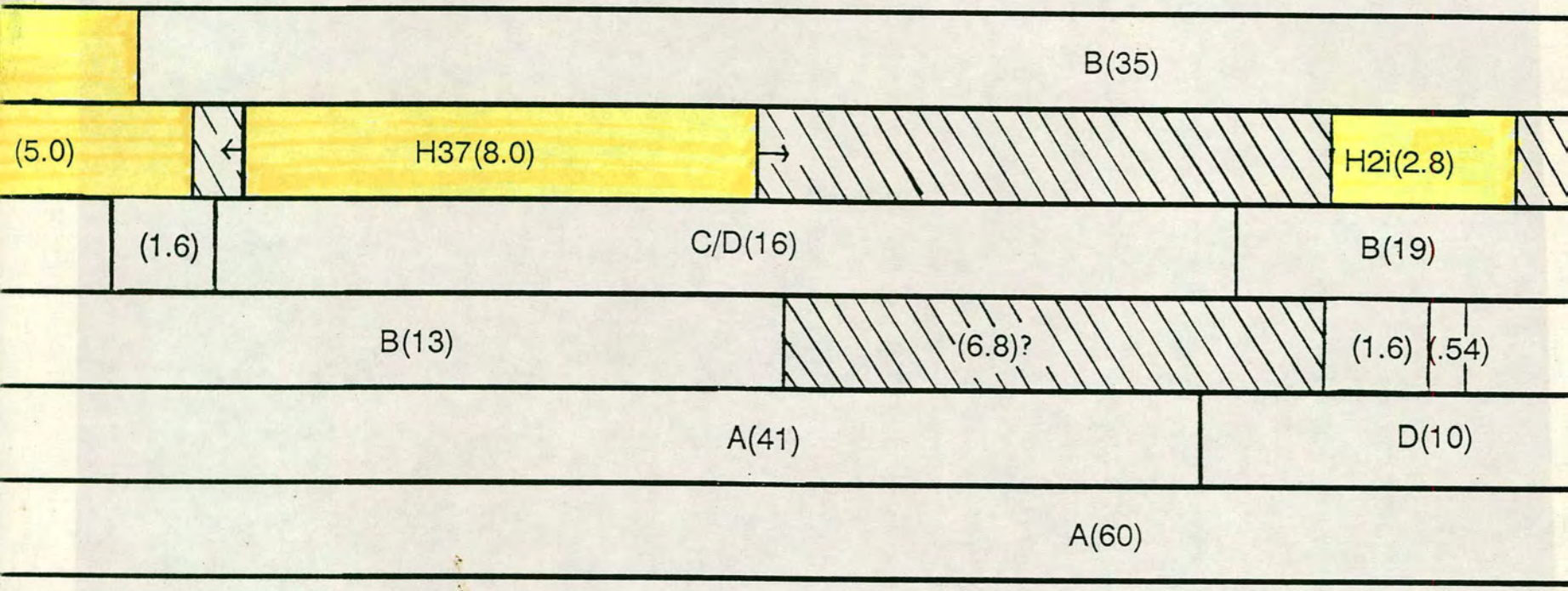
Sizes of the restriction endonuclease fragments are indicated in parentheses. Additional lettering indicates the clone designation or the relative fragment size, with fragments of decreasing size designated A-Z.

a)



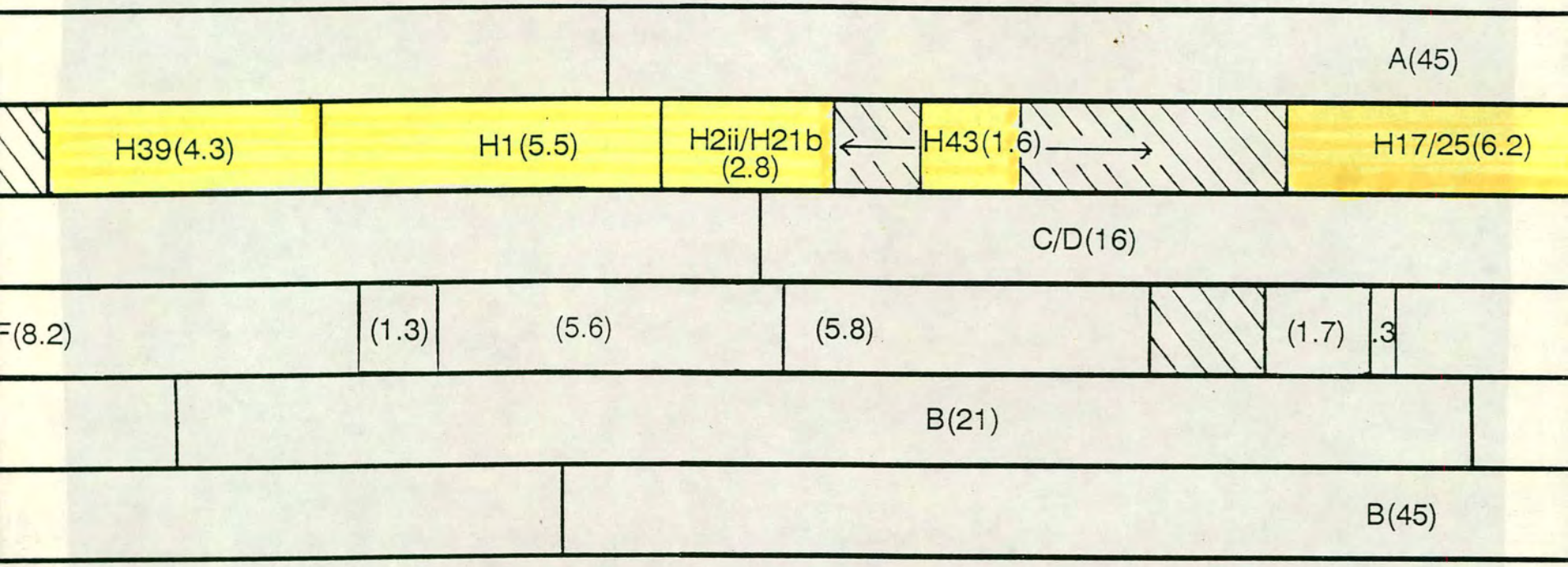
Junction of the unique and repeated DNA.

b)

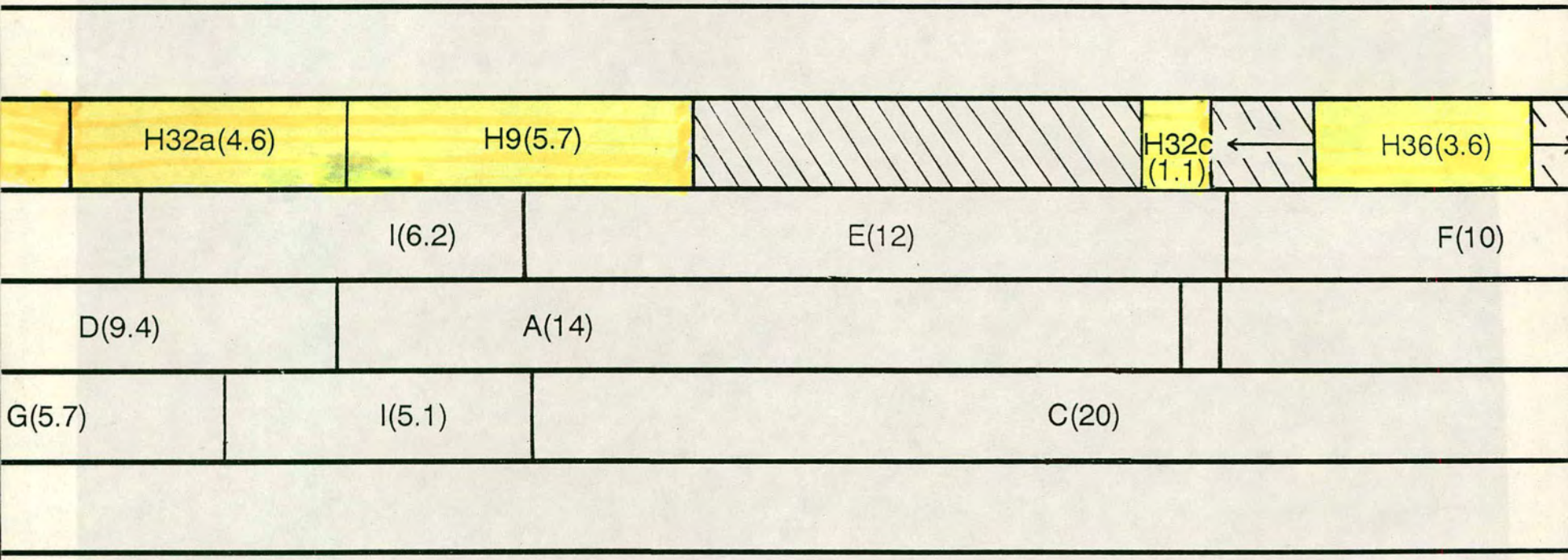


Region of uncertainty which could contain a 5.1 kbp *Bam*HI site, extending *Eco*RI -A to 40 kbp.

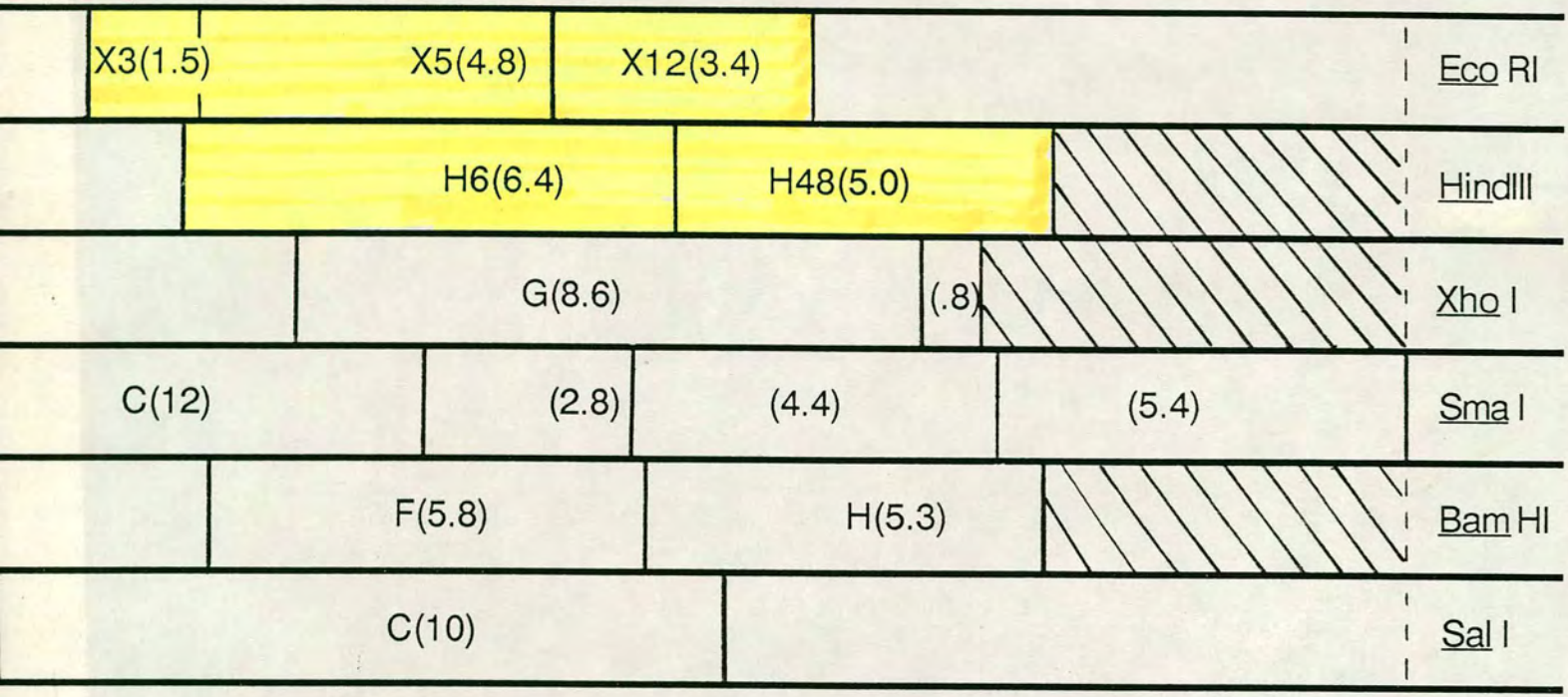
c)


A(45)
H39(4.3)
H1(5.5)
H2ii/H21b
(2.8)
H43(1.6)
H17/25(6.2)
C/D(16)
F(8.2)
(1.3)
(5.6)
(5.8)
(1.7)
.3
B(21)
B(45)

d)



e)



Junction of the unique and repeated DNA.

CHAPTER 4

DETECTION OF THE AHV-1 GENOME IN AFFECTED ANIMALS.

4.0 Introduction.

This chapter describes the study of the presence and nature of the AHV-1 genome within tissues and cell lines derived from AHV-1 -affected animals. No work has previously been described on the nature of the AHV-1 genome in cultured lymphoblastoid cell lines but previous researchers have shown, using indirect immunofluorescence, that there is little evidence of viral antigen in the tissues of affected animals (Rossiter, 1980; Patel and Edington, 1980 and 1981).

WC11 DNA clones were used to detect viral DNA in two lymphoblastoid cell lines derived from C500-infected animals and in tissue sections and cultured lymphoid cells from rabbits affected with MCF following inoculation with C500. Six distinct WC11 unique region clones and the repeat sequence clone were used to detect viral DNA in total DNA extracted from one or both of the lymphoblastoid cell lines. The repeat sequence clone alone was used to detect viral DNA in the tissues and cultured lymphoid cells from C500-infected animals. It was anticipated that the detection of viral DNA by *in situ* hybridisation would provide a greater sensitivity and specificity than the antigen detection by indirect immunofluorescence discussed above and would also enable latent virus to be detected. Use of the repeat sequence clone M30 increased sensitivity even further, because of the large copy number (20-25) of this sequence per genome.

4.1 Detection of AHV-1 DNA in lymphoblastoid cell lines derived from C500-infected animals.

The two lymphoblastoid cell lines derived from C500 -infected animals were cultured as previously described (Reid *et al.*, 1983 and 1989a). BJ/465 was derived from a Wistar rat (Jacoby *et al.*, 1988a) while BJ/610 was derived from a rabbit. The cell line BJ/610 required an exogenous source of interleukin 2 (IL-2) in the culture medium while BJ/465 did not require this growth factor. Both cell lines

are capable of transmitting MCF to susceptible animals of the same species on experimental inoculation but no viral particles or antigens have been detected in them (Reid *et al.*, 1983 and 1989a). However, virus has recently been recovered from BJ/610 by cocultivation (Reid, personal communication).

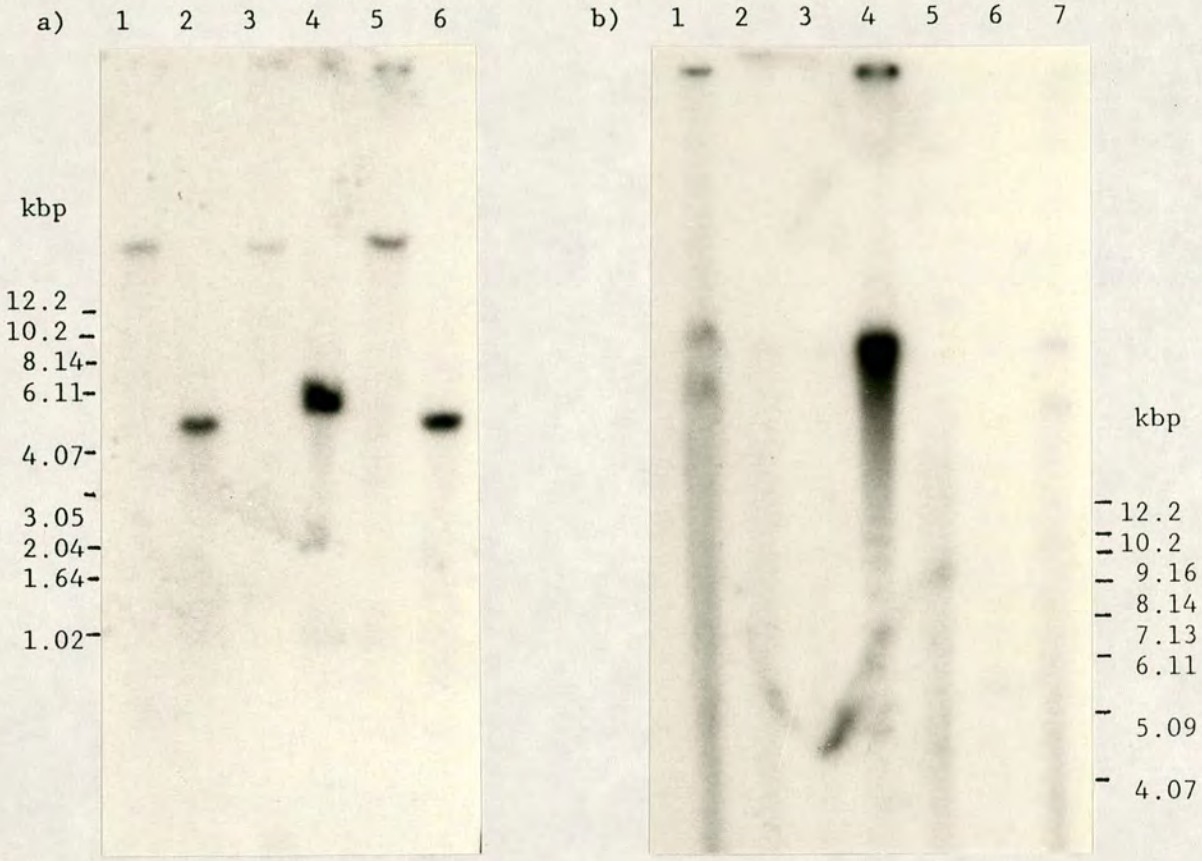
4.1.1 Detection of AHV-1 restriction endonuclease fragments.

Total DNA was prepared from approximately 10^7 cultured cells by SDS-proteinase K and phenol extractions. Samples were digested with the restriction endonucleases *Eco*RI or *Hin*dIII, electrophoresed through 0.5% agarose, transferred to nitrocellulose or to nylon membranes and used in hybridisation experiments with selected WC11 clones. The unique region clone R65 and the repeat sequence clone M30 both hybridised to fragments produced by *Hin*dIII and *Eco*RI digestion of BJ/465 DNA (Figure 4.1.1.1). The unique region clones R65, H1, H9, H17, X1, X5 and X12 and the repeat sequence clone M30 all hybridised to fragments produced by *Hin*dIII digestion of BJ/610 DNA; some of these results are shown in Section 5.1. The restriction fragments in BJ/610 DNA which hybridised to the WC11 clones all comigrated with similar fragments in C500 ca-infected BET DNA.

4.1.2 Detection of whole viral DNA in BJ/465.

Two methods were employed to determine whether virus within the lymphoblastoid cell line BJ/465 existed as episomal DNA or whether it was integrated into the chromosomes: Gardella gels (Section 7.4.3) and pulsed field gel electrophoresis (Section 7.4.2). No viral DNA could be detected using ^{32}P-labelled R65 or M30 probes on Southern blots of Gardella gels. Pulsed field gel electrophoresis of BJ/465 cells proved more successful. Fresh cells were embedded in agarose, total DNA extracted *in situ* and electrophoresed by crossed-field gel-electrophoresis. Figure 4.1.2.1 shows the autoradiogram produced following hybridisation of M30 to a Southern blot of total BJ/465 DNA. Hybridisation of M30 to a DNA fragment contained within

Figure 4.1.1.1
Autoradiograms showing hybridisation of ^{32}P-labelled WC11 clones a) p6f and b) M30 to total lymphoblastoid cell line DNA.



Track 1. BJ/437 *Eco*RI
2. BJ/437 *Hin*dIII
3. BJ/465 *Eco*RI
4. BJ/465 *Hin*dIII
5. BJ/465 *Eco*RI
6. BJ/465 *Hin*dIII

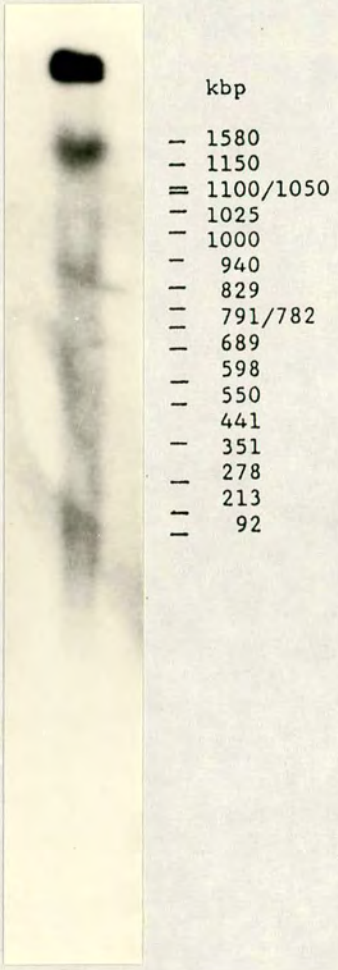
1. BJ/465 *Eco*RI
2. Uninfected rat LN *Eco*RI
3. BJ/437 *Eco*RI
4. WC11 *Eco*RI
5. Uninfected rat LN *Eco*RI
6. MF/629 *Eco*RI
7. BJ/465 *Eco*RI

BJ/437 and BJ/465 are lymphoblastoid cell lines derived from a C500-infected rat and rabbit respectively; BJ/437 was a short term culture and is therefore not described in the text. MF/629 is a lymphoblastoid cell line derived from a SA-MCF-affected cow and is described in Chapter 5. LN=lymph node.

Note the ladder of fragments produced by hybridisation of the repeat sequence clone M30 to *Eco*RI-digested WC11 DNA in b)track 4

Figure 4.1.2.1

Autoradiogram showing hybridisation of the WC11 unique region clone R65, labelled with ^{32}P-dATP, to DNA from the lymphoblastoid cell line BJ/465 separated by pulsed-field electrophoresis.



BJ/465 DNA is most pronounced in the region of the gel corresponding to DNA fragments of greater than 1000 kbp, which is likely to represent integrated DNA. No hybridisation was detected to DNA extracted from a normal rat lymph node cell suspension (not shown).

4.2 Detection of AHV-1 DNA in tissues derived from C500 -infected animals by *in situ* hybridisation.

Eight rabbits inoculated intravenously and two rats inoculated intraperitoneally with C500-infected cells were killed at the onset of clinical symptoms. Small (2mm) fragments of tissue were dissected, fixed and processed and peripheral blood leucocytes isolated as described in Section 7.6. These were assessed for the presence of virus by their infectivity and by *in situ* hybridisation. The WC11 repeat sequence clone M30 was used to probe sections of, firstly, rat kidney, heart, liver, lung and mesenteric lymph node and, secondly, rabbit kidney, liver, lung, spleen, appendix and submandibular and popliteal lymph nodes and peripheral blood leucocytes for the presence of viral DNA. Sections from one uninfected rabbit were also examined. Uninfected and WC11- or C500-infected cultured bovine thyroid cells were used as hybridisation controls.

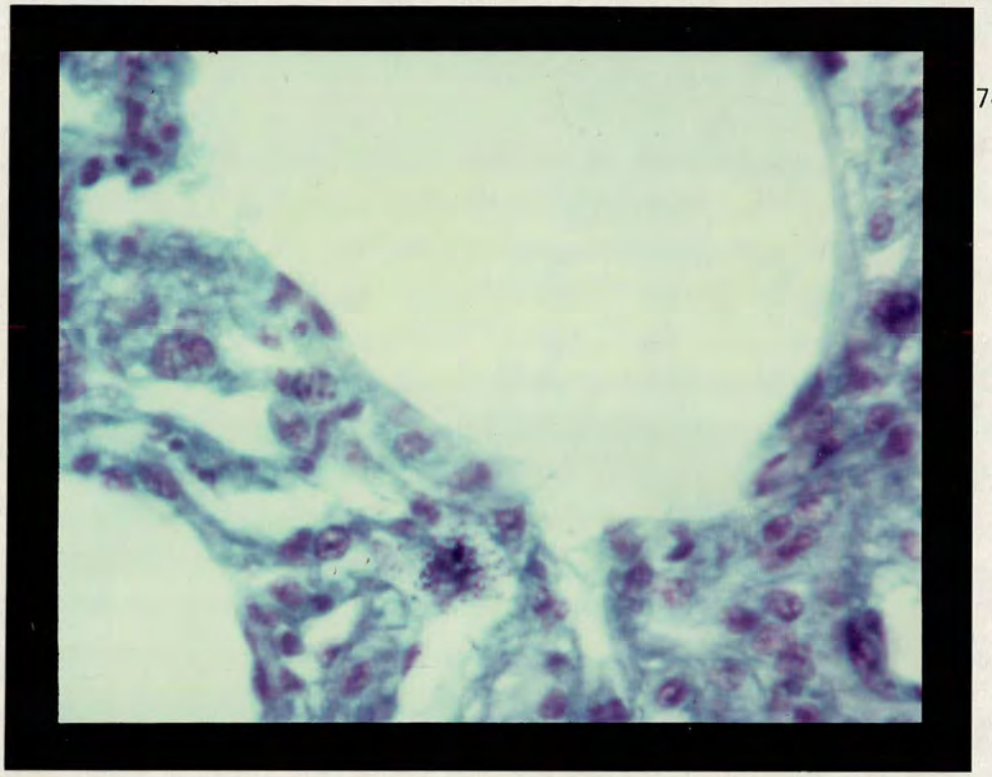
Estimates of viral infectivity for the rabbit tissues are shown in Table 4.2.1. These figures indicate that virus was present in almost all the tissues examined.

With the exception of rabbit RM88/3 sections, which were probed with ^{35}S-labelled M30 probe, all the sections were initially probed with ^{32}P-labelled M30. Further sections were prepared from those tissues showing cells positive for viral DNA by *in situ* hybridisation and from all the lymph nodes for probing with ^{3}H-labelled M30. The much lower energy of the particles produced by ^{3}H (maximum energy 18.6 keV) than by ^{32}P (maximum energy 1710 keV) produced results with a much higher resolution, but 13 week exposure times were required to compensate for the low frequency of

Rabbit ref no.	Death day pi.	Infectivity ($log_{10}TCID_{50}$/ 10^6 cells) lung	kidney	LN	spleen	PBL
RM88/7	8 (died)	1.16	<1.05	<1.3	2.35	NT
RM88/24	10	3.17	1.9	2.47	2.98	1.97
RM88/25	10	2.55	2.56	4.32	2.09	1.67
RM88/26	11	2.14	0.79	2.18	3.15	1.60
RM88/27	11	2.73	<1.0	2.89	2.48	2.03
RM88/28	12	3.13	1.38	2.84	3.92	1.69
RM88/29 (control)		-ve	-ve	-ve	-ve	-ve

Table 4.2.1

Infectivity recovered from tissues of rabbits terminally affected with AHV-1. The lymph nodes (LN) were popliteal. PBL = peripheral blood leucocytes.



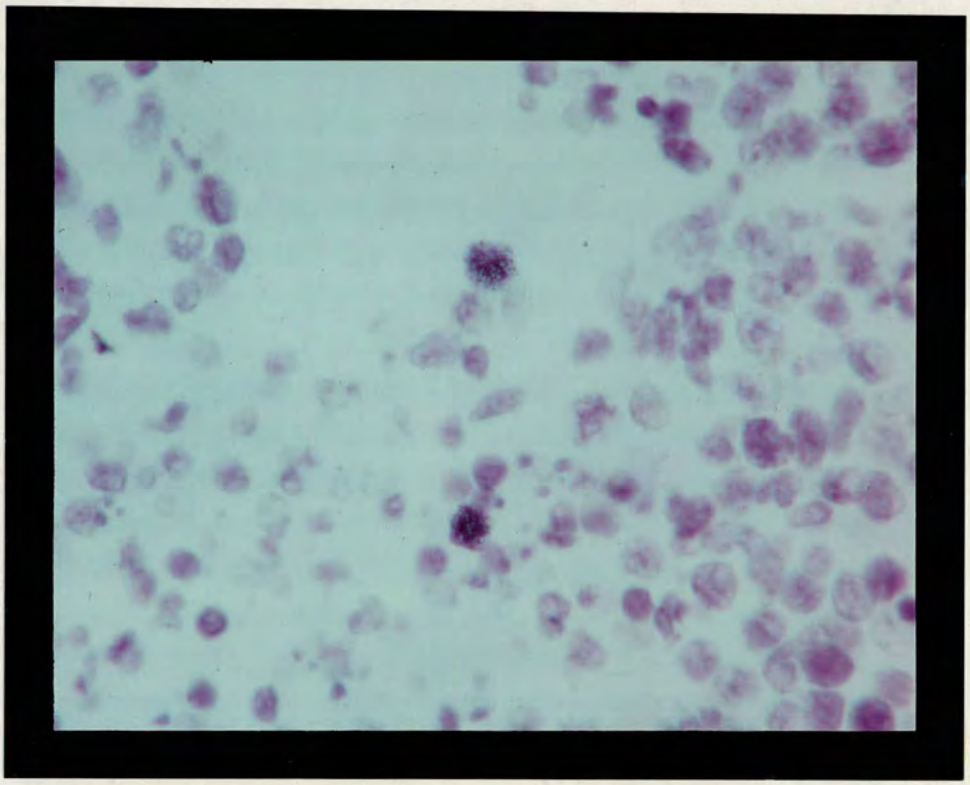


Figure 4.2.1. Giemsa-stained sections from C500-infected rabbits.

a) Lung derived from RM88/7, which died at 8 days p.i. The section was probed with ^{32}P-labelled M30, and exposed for 6 days. One positive cell is visible.

b) Submandibular lymph node derived from RM88/26, which was killed 11 days p.i. The section was probed with ^{3}H-labelled M30 and exposed for 13 weeks. Two positive cells are visible.

disintegration and the low efficiency of grain development (approximately 0.1 grain per disintegration for ^{3}H, Haase *et al.*, 1984).

The results showed an absence of AHV-1 -infected cells as detected by *in situ* hybridisation. The 2 rats examined showed signs of disease with multiple lesions, but no viral DNA was detected in the tissues. Consistently positive results were only obtained for two tissues: the lung of rabbit RM88/7 and the submandibular lymph node of rabbit RM88/26 (Fig. 4.2.1). Numerous positive cells were seen in some of the perivascular lymphoid aggregates in the lung of RM88/7; 34 and 67 AHV-1-infected cells were detected in the two sections probed with ^{3}H-labelled M30. However, no AHV-1 -infected cells were detected elsewhere in this animal, which became pyrexic at 5 days p.i. and died at 8 days p.i. It is possible that these cells represent a remainder of the initial inoculum (see Conclusion).

Triplicate experiments showed the presence of AHV-1 -infected cells in submandibular lymph node sections of RM88/26 following hybridisation with ^{3}H-labelled M30 probe and exposure for 13 but not for 6 or 10 weeks. No such infected cells were detected following hybridisation using a phosphorylated probe. Fig. 4.2.1b shows the result of hybridisation of ^{3}H-labelled M30 probe to RM88/26 cells. Four positive cells were detected in the lymph node section, which included a total of 91,000 cells giving a ratio of 1:23,000 virus infected: uninfected cells (ie., $\log_{10} 3.16/10^{6}$ cells) determined by *in situ* hybridisation. Two positive cells were detected in an adjacent section.

Positive results were obtained in the paracortical regions of some but not all lymph node sections examined of two more rabbits. Rabbit RM88/3, which was probed with ^{35}S -labelled M30, showed positive cells in one section. Rabbit RM88/25 showed no positive results with ^{32}P-labelled M30, but showed 2 positive cells with ^{3}H-labelled M30 following the 6 but not the 13 week exposure time.

Table 4.3.1

Details of the derivation and processing of the lymphoid cells cultured from C500-affected rabbits.

Rabbit ref no.	Death day pi.	Culture times (h)
BJ/597	10	0,24
BJ/643	11	0,2,4,6,8,10,12,14,16 24,28,32,36,48,72
BJ/644	17	0,2,4,6,8,10,12,14,16 24,28,32,36,48,72
BJ/663	11	0,3,6,12,24,48
BJ/664	12	0,3,6,12,24,48

Table 4.3.2

The infectivity and presence of AHV-1 DNA in cultured cells derived from the popliteal lymph nodes of 4 MCF-affected rabbits following 0 -72h of culture. The figures show the number of cells found positive for viral sequences by *in situ* hybridisation per cytospin (approximally 4×10^5 cultured cells).

Time (h)	BJ/643 -IL2	BJ/643 +IL2	BJ/643 inf	BJ/644 -IL2	BJ/644 +IL2	BJ/644 inf	BJ/663 -IL2	BJ/664 -IL2
0	0,0,x	-	<1.1	-	-	2.9	0	0
2	0,0,0	-	-			-	-	-
3	-	-	-	-	-	-	0	0
4	0,0,0	-	-	-	-	-	-	-
6	0,0,0	-	<1.1	-	-	2.7	0	0
8	0,0,0	-	-	-	-	-	-	-
10	0,0,0	-	-	-	-	-	-	-
12	0,0,10	-	<1.1	-,3	-	2.7	0,1	0,1
14	0,0,4	-	-	0,x,0	-	-	-	-
16	3,0,16	-	-	0,6,3	-	-	-	-
24	18,3	10,54	<1.1	3,5	x,4	3.4	0,6	x,3
28	4,4	-	-	2,10	-	-	-	-
32	46,5	-	-	0,3	-	-	-	-
36	x ,36	-	-	x ,0	-	-	-	-
48	0,x	46,x	2.6	0,6	14,9	2.8	0,x	0,0
72	65,44	99,216	3.0	x ,5	38,46	3.7	-	-

inf = infectivity recovered ($\log_{10}TCID_{50}$/ 10^6 cells)
- = not examined
x = interpretation difficult
-IL2/+IL2 indicates that cell culture was performed in the absence/presence of interleukin-2 respectively.

4.3 Detection of AHV-1 DNA in cultured lymphoid cells by *in situ* hybridisation.

Following the largely negative results with the *in situ* hybridisation experiments performed on rabbit sections, the presence of viral DNA in cultured lymphoid cells derived from C500-infected rabbits was assessed by *in situ* hybridisation. No virus could be detected in the supernatant fluid from such cultures (Reid, personal communication). Similar experiments using WC11-specific monoclonal antibodies showed that viral antigen could only be detected in lymphoid cells following at least 24h of culture (R. Munro, personal communication). Again, the *in situ* hybridisation results were compared with those for viral infectivity.

Five rabbits were employed in these experiments. The popliteal lymph nodes were collected on the second day of pyrexia and cells cultured *in vitro* for up to 48h (rabbits BJ/663 and BJ/664) or 72h (rabbits BJ/643 and BJ/644). The majority of cells were cultured in the absence of IL-2 but some cells taken from rabbits BJ/643 and BJ/644 were also cultured in the presence of IL-2. Samples of these cells were taken following 24, 48 and 72h of culture. Samples of approximately 4×10^5 cultured cells were taken for cytospin preparation at the time intervals indicated in Table 4.3.1. They were probed using ^{32}P-labelled (4 rabbits) or ^{35}S -labelled (rabbit BJ/597) M30 probe.

The results are shown in Table 4.3.2. No cells appeared positive for viral DNA by *in situ* hybridisation prior to 12h of culture, but a small number of positive cells were observed in some but not all cytospins prepared from all 4 rabbits following 12h of culture. The number of positive cells increased markedly on increased culture time from 12 to 48 or 72h. The silver grain density associated with the AHV-1 -infected cells also increased with time, but was still considerably lower than that of the WC11 and C500 -infected BT cells. The increase in virus-infected cells

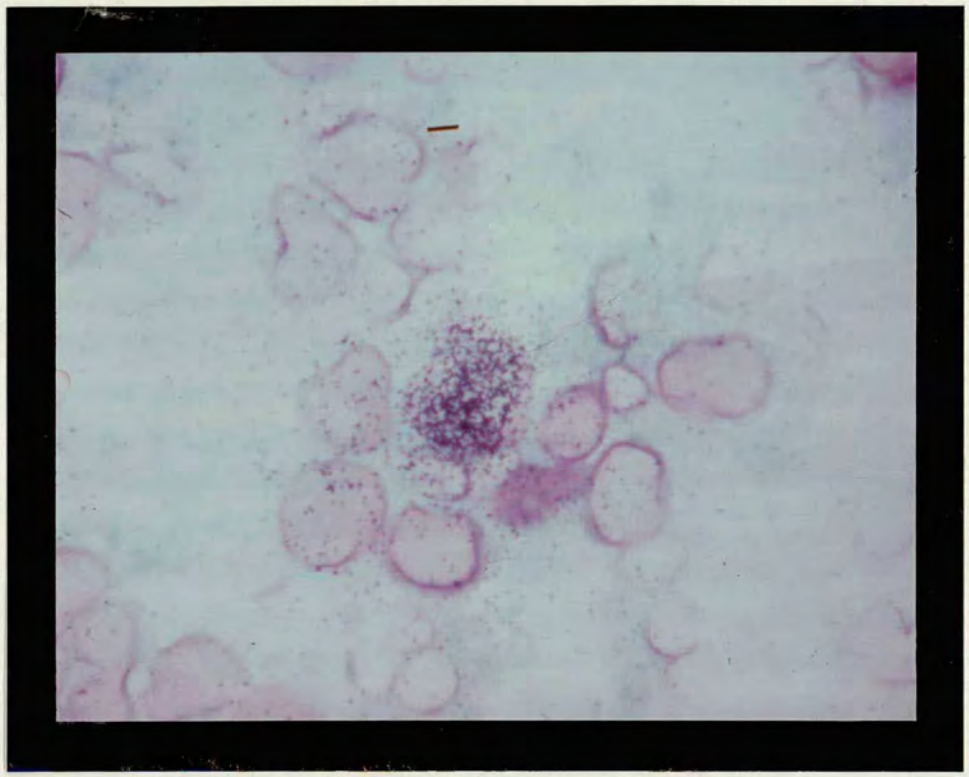


Figure 4.3.1
Giemsa-stained cultured BJ/644 cells probed with ^{32}P-labelled M30.
The cells were cultured for 28h and the slide was exposed for 6 days.

with time was particularly noticeable for the 2 cultures which were treated with IL-2. Table 4.3.2 shows the corresponding infectivity results for the cultured lymphoid cells. The figures show an increase in infectivity of approximately 100 fold for BJ/643 and of approximately 10 fold for BJ/644 on culture of the cells from 12 to 72h. This compares with increases of approximately 50 or 150 fold more positive cells for BJ/643 and 1 or 40 fold more positive cells for BJ/644 when cultured without or with IL-2 respectively over the same time period.

Fig. 4.3.1 shows BJ/644 cells cultured for 28h and probed with ^{32}P-labelled M30, and is typical of the results obtained in these experiments. The single cell positive for viral DNA is covered in more than 200 silver grains, while neighbouring cells are covered by between 0 and 30 grains. Uninfected cells further from positive centres, which were unaffected by the scatter of the high energy particles, had grain counts of 0-5 per cell. The high energy of the particles precluded viral DNA localisation to the nucleus or cytoplasm.

4.4 Conclusion.

Analysis of the lymphoblastoid cell lines BJ/465 and BJ/610, which were derived from AHV-1 -affected animals, clearly demonstrated the presence of viral DNA. The seven WC11 unique region clones used to probe total DNA extracted from BJ/610 cells and C500-infected cells indicated that there was no rearrangement of the regions of the viral genome assessed in that cell line. Pulsed-field gel-electrophoresis suggested that viral DNA could be integrated in the cell line BJ/465. This cell line, unlike BJ/610, behaves as a transformed cell line in its growth characteristics and requirements, and the AHV-1 genome could therefore exist in a different form in these two cell lines.

The *in situ* hybridisation results confirm and extend earlier work by showing the singular lack of virus in

tissues and in cultured lymphoid cells taken from rabbits clinically affected with MCF.

The results obtained for the detection of viral DNA in the tissues of MCF-affected animals by *in situ* hybridisation indicated that virus-infected cells were present at a ratio of approximately $1:10^4$. This is an estimate approximately 100 fold higher than that obtained by Patel and Edington for experimentally infected rabbits and cattle by indirect IIF (1980 and 1981). However, the observation of viral infectivity in tissues for which the *in situ* hybridisation results were negative showed that low copy number viral genomes were still not being detected. Use of a riboprobe could have provided the sensitivity to indicate the proportion of infected cells more precisely (Cox *et al.*, 1984), but the importance of these results lies in the confirmation of the low levels of viral DNA in the cells of dying animals using a reliable detection system. This finding means that viral cytolysis is an unlikely explanation for the pathogenesis of MCF but it is consistent with the hypothesis that the lesions associated with MCF arise through a cell-mediated immune dysfunction induced by AHV-1 (Reid and Buxton, 1984).

The lymphoid cells cultured from C500-affected rabbits showed an increase in viral infectivity and in the number and level of virus-infected cells with time. That de-repression occurred *in vitro* suggests that inhibition to replication is a host-maintained state. A similar de-repression may have occurred in rabbit RM88/7; it is possible that the cells in the inoculum could have remained in this rabbit and de-repression occurred as in an *in vitro* culture. Similar results have been obtained by Patel and Edington (1982), who demonstrated the presence of virus-specific antigen on the surface of lymphocytes cultured from AHV-1 -affected rabbits following 24h of culture.

CHAPTER 5

DETECTION AND IDENTIFICATION OF THE VIRAL AGENT OF SA-MCF.

5.0 Introduction.

There is good evidence that there are at least two causative agents of MCF and that sheep are carriers of a viral agent which is antigenically related to AHV-1 (Section 1.1.2.2). No virus has yet been cultured from sheep or from SA-MCF -affected animals which is capable of inducing MCF in susceptible species, as discussed previously. Reid *et al.*, (1985 and 1989b) have derived numerous lymphoblastoid cell lines from the tissues of both AHV-1 and SA-MCF -affected animals and these have been employed in the assessment of the relationship between AHV-1 and the agent of SA-MCF.

The lymphoblastoid cell lines employed in these experiments are descibed in Table 5.0.1. They were derived from a range of tissues from a variety of species clinically affected with MCF and vary in their dependence upon IL-2 and ability to transmit disease. No viral particles have been observed in the cell lines by electron microscopy, and no viral antigen has been detected (Reid *et al.*, 1983, 1989b). The NK-like activity of these cell lines has been referred to previously (Section 1.1.5). Analysis of the cell lines with monoclonal antibodies directed against different cell markers has shown that the bovine cell lines possess T4 or T8 antigens while those derived from deer possess T19 antigen (which may be associated with NK-like activity) or were not recognised by the monoclonals (Reid, personal communication). The different lymphocyte subtypes thus infected by the SAA may explain the different properties of the cell lines.

5.1 Detection of sequences homologous to WC11 DNA in lymphoblastoid cell lines derived from SA-MCF -affected animals.

Total DNA was isolated from approximately 5×10^7 cells from each of the cell lines by SDS-proteinase K and phenol extractions and concentrated by ethanol precipitation. Following restriction endonuclease digestion, fragments were separated through 0.5% agarose in TBE buffer, transferred to

Table 5.0.1

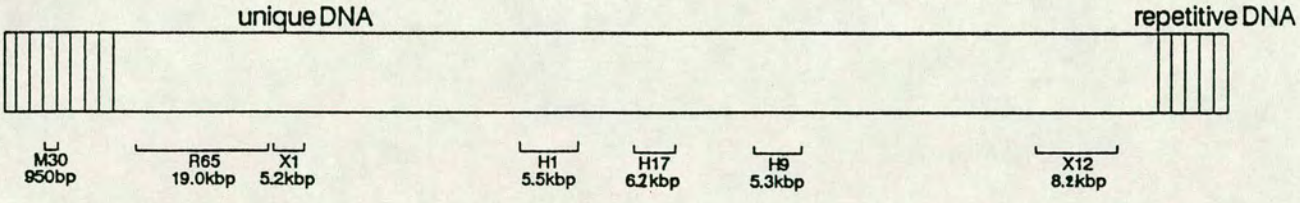
Source and characteristics of the lymphoblastoid cell lines derived from animals with malignant catarrhal fever.

CSF - cerebrospinal fluid; LN - lymph node.

Cell line	Species	Tissue	Period in culture	Agent	IL-2 dependence	Disease transmission
MF/629	Bovine	CSF	5 years	SAA	-	-
BJ/576	Bovine	Cornea	1 year	SAA	-	-
MF/816	Red deer	LN	3 years	SAA	+	+
MF/824	Red deer	LN	5 months	SAA	+	-
BJ/615	Red deer	LN	9 months	SAA	+	+
BJ/393	Rabbit	LN	7 months	SAA	-	+
BJ/610	Rabbit	LN	9 months	AHV-1	+	+
BJ/465	Rat	LN	1 year	AHV-1	-	+

Figure 5.1.1

The genomic location and insert sizes of the seven AHV-1 clones used to assess the homology with the SAA.



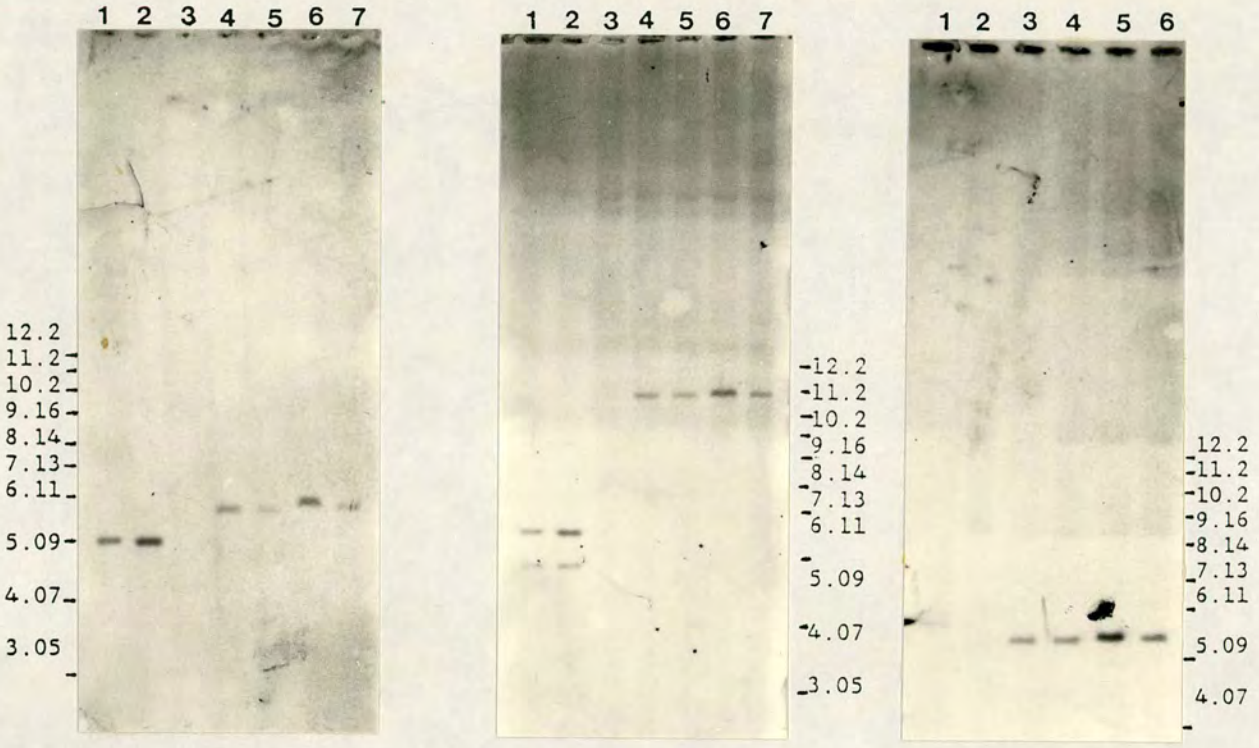


Figure 5.1.2
The figure shows hybridisation of a) clone H1, b) clone X12 and c) clone H17 to total DNA extracted from the SA-MCF cell lines BJ/576, BJ/615, MF/629 and MF/816.

a),b) Track 1: BJ/610; track 2: C500-infected BT; track 3: BET; track 4: BJ/615; track 5: BJ/576; track 6: MF/629; track 7: MF/816. c) Track 1: C500-infected BT; track 2: BET; track 3: BJ/615; track 4: BJ/576; track 5: MF/629; track 6: MF/816.

DNA extracted from C500 infected bovine thyroid (BT) cells and from the AHV-1 MCF cell line BJ/610 was used as a positive hybridisation control while DNA extracted from uninfected bovine embryonic thyroid (BET) or testes (BETe) cells was used as a negative control. The DNAs were digested with *Hin*dIII, electrophoresed through 0.5% agarose and transferred to nitrocellulose. The clones were labelled with digoxygenin-dUTP and bound probe was detected using alkaline -phosphatase conjugated sheep polyclonal anti -digoxygenin.

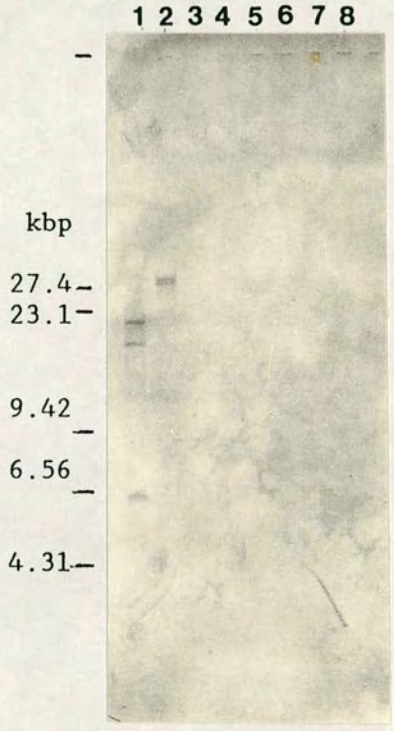
nitrocellulose or nylon membranes and hybridised with WC11 clones labelled with ^{32}P-dATP or with digoxygenin-dUTP. Six different unique region WC11 clones: H1, H9, H17, X1, X12 and R65 and the repeat sequence clone M30 were used in the hybridisation experiments. The unique region clones were derived from different regions of the WC11 genome as indicated in Figure 5.1.1

There was detectable homology between the AHV-1 clones H1, H9, H17, X12 and R65 and DNA extracted from the six cell lines derived from animals with SA-MCF. Figure 5.1.2.a), b), and c) shows hybridisation of clones H1, X12, and H17 to DNA extracted from the SA-MCF cell lines BJ/576, BJ/615, MF/629 and MF/816, but not to DNA extracted from uninfected cultured bovine cells under conditions of medium stringency. H9 (not shown), H17 and X12 each hybridised to a single fragment, which differed in size with the probe used, but which was identical in size in the DNAs from all of the cell lines derived from SA-MCF -affected animals regardless of their origin, ability to transmit disease or dependence upon IL-2. In each case, the molecular weight of this fragment differed from that of the cross-hybridising C500 fragment. The single SA-MCF viral fragment which hybridised to H1 (Fig. 5.1.2a) showed slight variation in molecular weight between the cell lines, while that homologous to R65 showed marked variation (not illustrated). R65 also hybridised to sequences contained within the AHV-1-derived cell line BJ/465 and to the SA-MCF-derived cell lines BJ/393 and MF/824 (not shown).

Neither the vector probe, the unique region clone X1, nor the repeat sequence clone M30 hybridised to sequences within the SA-MCF -derived lines under conditions of medium stringency (50% formamide and 4 x SSC at 42°C). The hybridisation of X1 to the SA-MCF cell lines under conditions of low stringency was not assessed. Low stringency hybridisations were not applicable for use with M30 because the extremely high G+C content of the probe

Figure 5.1.3
Hybridisation of the AHV-1 repeat sequence clone M30, labelled with digoxygenin-dUTP, to *Eco*RI and *Hin*dIII digests of total lymphoblastoid cell line DNA and to control bovine embryonic brain (BEB) DNA.

Track 1. BJ/610 *Hin*dIII
2. BJ/610 *Eco*RI
3. BJ/576 *Hin*dIII
4. BJ/576 *Eco*RI
5. BEB *Hin*dIII
6. BEB *Eco*RI
7. BJ/615 *Hin*dIII
8. BJ/615 *Eco*RI



(72%) would have led to spurious results. In contrast, M30 hybridised to two *Eco*RI DNA fragments in both the AHV-1 -derived MCF cell lines BJ/465 and BJ/610 under conditions of medium stringency (Fig. 4.1.1.1 and Fig. 5.1.3).

No hybridisation was observed when clone R65 was used to probe total DNA extracted from sheep peripheral blood lymphocyte DNA (not shown).

5.2 Derivation of the putative SAA clones from the lymphoblastoid cell line MF/629.

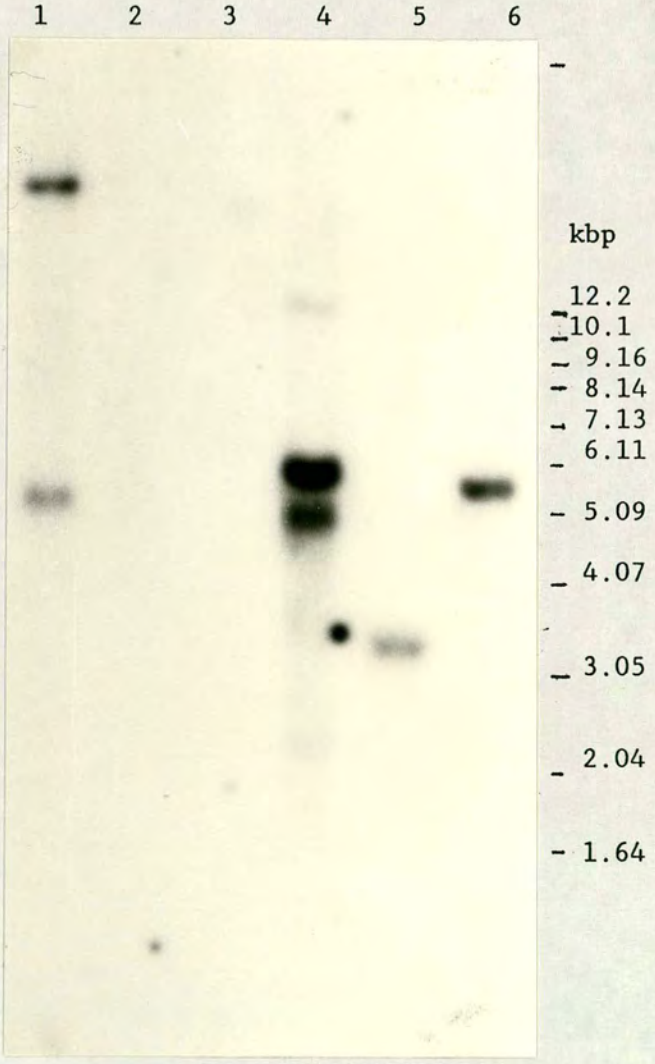
A genomic library of the lymphoblastoid cell line MF/629 DNA was constructed in λNM1149 in an attempt to isolate DNA sequences corresponding to the SAA. The MF/629 cell line was chosen because of the ease with which the cells could be grown, compared with the other cell lines, and because hybridisation experiments had indicated an abundance of putative SA-MCF viral DNA. The enzyme *Hin*dIII was chosen for the cloning following hybridisation of the AHV-1 clone R65 to a blot of MF/629 DNA digested with the endonucleases *Eco*RI, *Hin*dIII, *Sac*I and *Sma*I; *Hin*dIII produced three fragments with homology to R65 in the 0-9kbp range (Figure 5.2.1).

*Hin*dIII subclones of R65 were constructed in pUC13 for use as probes to screen the genomic library. The pUC13 subclones p4e (equivalent to clone H10), p6a (equivalent to clone H26) and p6f (equivalent to clone H21a/H22) with inserts of 8.0, 5.2, and 4.4 kbp respectively were described in Chapter 3. No hybridisation to MF/629 DNA was detected with p4e or p6f but the subclone p6a was found to hybridise to the same *Hin*dIII and *Eco*RI fragments of MF/629 DNA as the parent clone R65. The pUC13 clones did not hybridise to λNM1149 DNA.

Figure 5.2.1

Autoradiogram showing hybridisation of the AHV-1 clone R65 (labelled with ^{32}P-dATP) to digests of MF/629 DNA and to control bovine DNA.
BET = bovine embryonic thyroid.
BEK = bovine embryonic kidney.

Track 1. MF/629 *Eco*RI
2. BET *Eco*RI
3. BEK *Eco*RI
4. MF/629 *Hin*dIII
5. MF/629 *Sma*I
6. MF/629 *Sac*I



The hybridisation of p6a to three distinct *Hin*dIII fragments of 5-7 kbp in MF/629 DNA could be explained by the presence of repeated sequences in the genomes of both viral agents or by rearrangement and/ or duplication of the SAA sequences in the cell line. Evidence indeed suggests that sequence duplication of the SAA genome may have ocurred in MF/629 (see Conclusion) and that clone p6a, at least, possesses internal repeated sequences (see Section 2.4).

MF/629 and λNM1149 DNA were restricted with *Hin*dIII, ligated and packaged. Phage were plated at a density of 6-8,000 pfu per plate using the *rec*A+ *hfl*- strain NM533. Phage DNA was transferred to nylon membranes and probed with p6a. From the 50,000 or so plaques analysed, twelve plaques were obtained which showed weak hybridisation to p6a. Four of these were plaque-purified four times using NM533 as host; the plaques maintained their characteristic weak hybridisation to p6a. DNA was prepared from three clones, designated λ8a, λ9a and λ12b. λ9a possessed an insert of 5.7 kbp while λ8a and λ12b possessed dual inserts of 5.0 and 3.5 kbp and of 5.0 and 4.0 kbp, respectively. p6a hybridised to the larger inserts of both λ8a and λ12b.

5.3 Hybridisation properties of the SAA clones.

The validity of the SAA clones was assessed by hybridisation to AHV-1 DNA and to total DNA extracted from lymphoblastoid cell lines derived from AHV-1 and from SA-MCF -affected animals. ^{32}P-labelled λ9a showed strong hybridisation to DNA extracted from the cell lines MF/816 and MF/824, derived from SA-MCF -affected animals, but only weak homology to AHV-1 DNA under conditions of medium stringency. The clone showed strong hybridisation to the larger inserts of clones λ8a and λ12b under similar conditions. Digoxygenin-labelled λ8a hybridised to single fragments produced by *Hin*dIII digestion of BJ/576, BJ/615 and MF/816 DNA and to two fragments produced by *Hin*dIII digestion of MF/629 DNA, but not to DNA extracted from BJ/610 or to C500 DNA under the conditions recommended for

Figure 5.3.1
Hybridisation of the digoxygenin-labelled putative SAA clone λ8a to digests of total lymphoblastoid cell line DNA derived from animals affected with C500 or with SA-MCF. Development time was only 5 minutes.
BT = bovine thyroid.

Track 1. C500 ca-infected BT DNA *Hin*dIII
2. BJ/610 *Hin*dIII
3. BET DNA *Hin*dIII
4. BJ/576 *Hin*dIII
5. BJ/615 *Hin*dIII
6. MF/629 *Hin*dIII
7. MF/816 *Hin*dIII

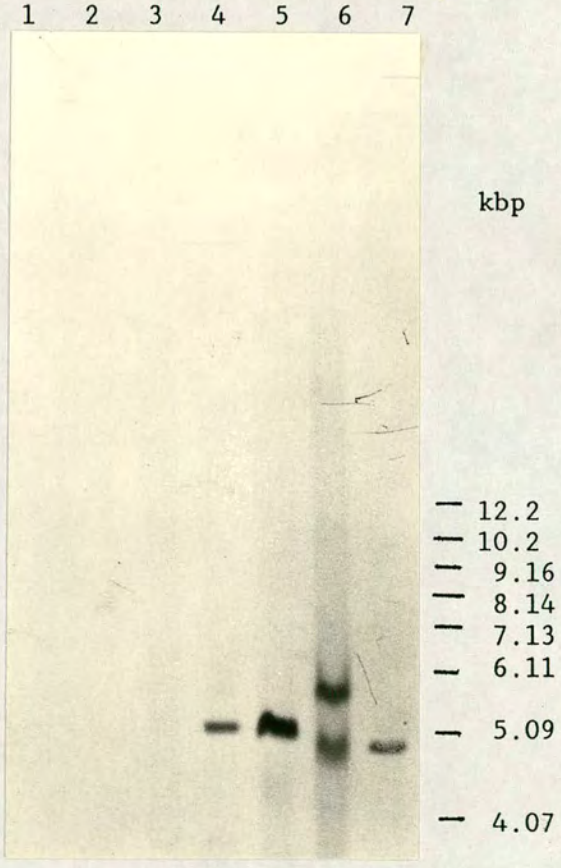
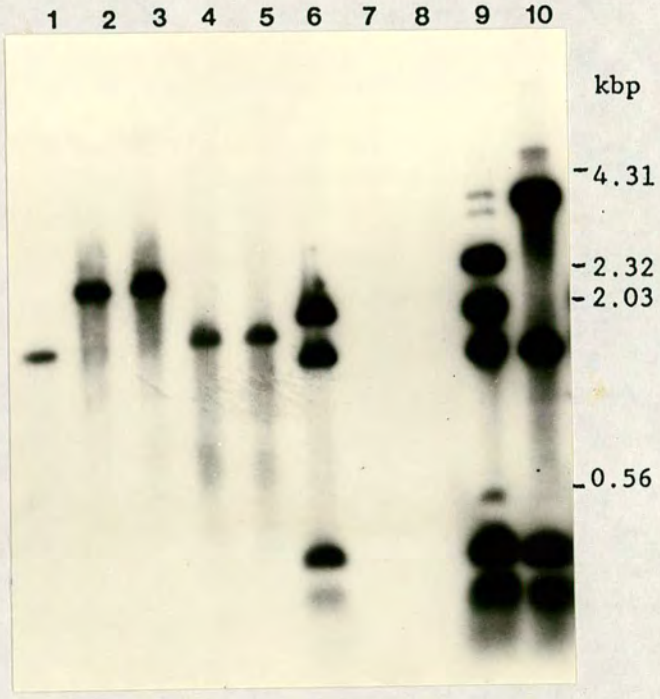


Figure 5.3.2

Autoradiogram showing hybridisation of the AHV-1 clone p6a (labelled with ^{32}P-dATP) to digests of AHV-1 clones and of the putative SAA clones.

R65 and its subclone p6a are WC11 clones, while λ8a, λ9a and λ12b are putative SAA clones.

Track 1. λ12b *Hin*dIII *Ava*I
2. λ9a *Hin*dIII *Ava*I
3. λ9a *Ava*I
4. λ8a *Hin*dIII *Ava*I
5. λ8a *Ava*I
6. R65 *Hin*dIII *Ava*I
7. (λNM1149 *Ava*I)
8. (λcI857 *Hin*dIII)
9. p6a *Hin*dIII *Ava*I
10. p6a *Ava*I



use with the Boehringer digoxygenin kit (Figure 5.3.1). Since the development time for the blot shown was only five minutes, no hybridisation was observed to C500 DNA; hybridisation of λ8a did occur under the same hybridisation conditions to larger amounts of WC11 DNA (not shown). A WC11 clone hybridised to fragments in the digested C500 and BJ/610 DNA in a parallel experiment (not illustrated). The sizes of the two MF/629 *Hin*dIII fragments which hybridised to λ8a corresponded to the insert sizes of the putative SAA clones λ8a and λ12b and to λ9a.

The SAA clones were compared with the corresponding AHV-1 clone p6a by restriction endonuclease analysis and hybridisation (Figure 5.3.2). The figure clearly illustrates the much stronger hybridisation signal obtained following hybridisation of p6a to itself than to equivalent loadings of the clones λ8a, λ9a and λ12b. p6a hybridised to *Ava*I fragments of 1.8 kbp in λ8a and λ12b DNA, and to a fragment of 2.5 kbp in λ9a DNA. Digestion of the clones with *Alu*I and *Hpa*II and hybridisation of the ensuing blots with p6a yielded similar results, with cross-hybridising fragments in λ9a DNA which were 700 bp larger than those in λ8a and λ12b DNA (not illustrated). These results indicated that the cross-hybridising regions of the WC11 and putative SAA clones were smaller than was evident following digestion with *Ava*I, since p6a hybridised to λ8a and λ12b *Hpa*II and *Alu*I fragments of 1.5 and 1.15 kbp respectively. The results just discussed, the identical insert sizes of λ8a and λ12b and the strong cross-hybridisation of the inserts of all three putative SAA clones suggest that clones λ8a and λ12b are identical, and are related to the larger λ9a clone.

5.4 Conclusion.

Previous researchers have shown that sera from sheep and from some SA-MCF -affected animals react with AHV-1 (Section 1.1.2.2). However, as AHV-1 shows some antigenic cross-reactivity with bovid herpesviruses 1, 2 and 4

(Rossiter *et al.*, 1977, 1988, 1989; Heuschele, 1982), an immunological cross-reactivity between the two putative MCF agents does not prove their similarity. The results described in Section 5.1 provide convincing confirmatory evidence for a relationship between AHV-1 and the agent of SA-MCF. Lymphoblastoid cell lines derived from SA-MCF -affected animals were used in this analysis because some of these lines transmit SA-MCF to susceptible animals and can therefore be assumed to contain the genome of the SA-MCF viral agent. Five distinct unique region clones of AHV-1, derived from well spaced regions of the genome, hybridised to total DNA extracted from these cell lines under conditions of medium stringency. No hybridisation was observed to control cultured bovine cells assessed concurrently.

The hybridisation results indicated that the SA-MCF agent contained within the lymphoblastoid cell lines varied only slightly between the cell lines. The slight variation could arise from strain variability or from gene rearrangements during culture. Gene rearrangements and deletions have been observed in episomal copies of *H.saimiri* in lymphoblastoid cell lines derived from tumours induced by this virus (Ablashi *et al.*, 1985; Desrosiers, 1981). It is possible that similar rearrangements in the viral sequences contained within the SA-MCF -derived lines have given rise to the variation in restriction fragment length observed following hybridisation with some of the AHV-1 clones and to the loss of disease transmissibility in three of the cell lines (Table 5.0.1). The derivation of two different but cross -hybridising clones from MF/629 DNA, one of which, λ9a, appears to contain similar but additional DNA to the other, λ8a/12b, suggests that there may have been sequence duplication of the SAA DNA in MF/629.

No attempt has been made to accurately measure the number of viral genome equivalents in the cell line MF/629. However, a rough estimate of approximately 3 copies per

cell can be calculated from the number of clones obtained in the MF/629 genomic library with homology to WC11 DNA (see Appendix 5).

The derivation of two different but cross-hybridising SAA clones should provide a means of detection of the SAA. This is discussed more fully in the general discussion in Chapter 6.

CHAPTER 6

GENERAL DISCUSSION.

6.1 Comparison of the malignant catarrhal fever viruses with the *H.saimiri*-like herpesviruses.

6.1.1 Biological properties.

Numerous authors have commented upon the similarities of the epidemiology and pathology of malignant catarrhal fever to that of the leukaemias and lymphomas induced by the *H.saimiri*-like herpesviruses (Hunt and Billups, 1979; Patel and Edington, 1980 and 1981; Plowright, 1982). All the viral agents are strictly cell-associated at low passage *in vitro* and *in vivo* following the initial infection. (Patel and Edington, 1980). Features of the pathogenesis of these infections are discussed below.

The viruses HVA, HVS, *H. aotus* type 2 and both AHV-1 and the agent of SA-MCF all infect their natural hosts in an asymptomatic manner, but induce disease in related species following natural infection (AHV-1: Plowright, 1968; *H.saimiri*-like herpesviruses: reviewed by Fleckenstein and Desrosiers, 1982). Other species can additionally be infected by experimental inoculation. The T-lymphotropic herpesvirus *H. sylvilagus* differs, however, from these viruses in inducing lymphoproliferative disease in its natural host, the cottontail rabbit (Hinze, 1971). Infection of the primary hosts of AHV-1, the agent of SA-MCF and HVS almost entirely occurs by horizontal transmission, usually in the first few months of life. Thus, most wildebeest (*Connochaetes taurinus*) of seven months of age are infected with AHV-1 (Plowright, 1968), while most free-living squirrel monkeys (*Saimiri sciurius*), the natural host of HVS, are infected by two years of age (Fleckenstein and Desrosiers, 1982). Following an acute subclinical infection the virus tends to remain latent in the host, probably for life, but can be reactivated by physical stress or by drug treatment. Both HVS and AHV-1 can also be reactivated *in vitro* by cocultivation of host lymphocytes with permissive cells.

Infection of susceptible animals other than the primary host by these viruses results in lymphoproliferative disease. The incubation period of the diseases, although normally short, can vary greatly. For example, the incubation period for MCF varies between 2 days and 10 months while that for disease induced by HVS varies from 2 weeks to 1 year (reviewed by Hunt and Billups, 1979). Infection of New World monkeys with HVS, HVA or *H.aotus* type 2 leads to the development of leukaemias and lymphomas (Fleckenstein and Desrosiers, 1982) while both forms of MCF are characterised by a T cell hyperplasia rather than a neoplastic response. MCF also involves degenerative lesions of the respiratory and alimentary tracts (Section 1.1.1) which are not observed in HVS, HVA and *H.aotus* -affected animals. However, the pathological mechanisms may still be similar, since AHV-1 is capable of inducing neoplasia in experimentally-infected rats (Jacoby *et al.*, 1988a and b). In addition, the lymphoblastoid cell lines derived from AHV-1 and SA-MCF -affected animals show the characteristics of transformed cells (discussed below).

Another feature common to AHV-1, the SA-MCF agent and the gammaherpesviruses is the ability of these viruses to transform or modify susceptible cells such that continuous lymphoblastoid cell lines can be derived. Lymphoblastoid cell lines have been derived from the tumours of HVS (Ablashi *et al.*, 1985; Fleckenstein *et al.*, 1977) and HVA -affected animals, and also by *in vitro*-infection of susceptible cells with HVS (Rabin *et al.*, 1984; Schirm *et al.*, 1984), HVA (Falk *et al.*, 1974) or *H.sylvilagus* (Kramp *et al.*, 1985). Similar cell lines have been derived from both AHV-1 and SA-MCF-affected animals (Reid *et al.*, 1983 and 1989b); these cell lines have been employed in this study (Chapter 5).

The characteristics of the lymphoblastoid cell lines from different hosts and immortalised by either the *H.saimiri*-like herpesviruses or the MCF agents are similar.

The cells commonly express T-cell markers (Neubauer *et al.*, 1981; Kiyotaki *et al.*, 1986 and 1988; Reid, personal communication) and, in all cases, the cells possess the morphology of large granular lymphocytes. The lymphoblastoid cell lines also possess natural killer cell-like activity; this has been clearly demonstrated for tumour-derived HVS and HVA cell lines (Johnson and Jondal, 1981), for cell lines derived by *in vitro* transformation with HVS (Ortaldo *et al.*, 1985; Kiyotaki *et al.*, 1986) or with HVA (Kiyotaki *et al.*, 1988) and for the cell lines derived from SA-MCF-affected animals (Reid *et al.*, 1983 and 1989b).

The ability of the cell lines to transmit disease varies both with the line and the period in culture, since some cell lines lose the ability to transmit disease after extensive culture. The dependence of the cell lines on the T-cell growth factor IL-2 also varies. Thus, some HVS-containing lymphoblastoid cell lines require this factor for growth (Ablashi *et al.*, 1985; Szomolanyi *et al.*, 1987), while not all cell lines derived from MCF-affected animals require this factor (Reid *et al.*, 1989b). By definition, therefore, some of the cell lines derived from MCF-affected animals are transformed; all appear to be immortalised.

Examination of lymphoblastoid cell lines from numerous sources has shown that they contain large numbers of episomal copies of the respective viral genome (Werner *et al.*, 1977 and 1978; Johnson *et al.*, 1981 and Kaschka-Dierich *et al.*, 1982). For example, HVS-containing lymphoblastoid cell lines contain approximately 100-300 viral copies (Fleckenstein and Mulder, 1980). Episomal viral genomes have also been observed in lymphoblastoid cell lines derived from EBV tumours (Adams and Lindahl, 1975). Viral deletions, duplications or rearrangements are common in cell lines containing HVS or HVA (Ablashi *et al.*, 1985; Desrosiers, 1981); in fact up to 73% of the HVS genome can be deleted in immortalised cells (Schirm *et al.*, 1984). The

viral genomes are thought to be in a latent state when episomal; Knust *et al.*, 1983a showed one HVS-derived cell line to contain only one transcript of 2.7 kbp, which was thought to correspond to the immediate-early transcript. There is also some evidence for viral integration in some of these HVS cell lines, but this does not appear to be the major state of the genome (Kaschka-Dierich *et al.*, 1976).

The approximate estimate of 3 viral genome equivalents in the lymphoblastoid cell line MF/629, which was derived from a SA-MCF -affected bovine (Section 5.0), is lower than that published for the HVS-containing cell lines. The hybridisation results shown in Chapter 5 for the other cell lines derived from SA-MCF -affected animals provided the first evidence for viral DNA within these cell lines and indicated a similar viral copy number. The work described in Chapter 4 also provides the first evidence that the lymphoblastoid cell lines BJ/465 and BJ/610, which were derived from C500-affected animals, contain AHV-1 DNA.

The experiments described in Chapter 4 demonstrate the ability of AHV-1 to be reactivated on *in vitro* culture of cells taken from AHV-1 -infected animals, thus indicating that the virus had been present in a latent form within these cells. This is a well documented finding for HVS-infected cells (reviewed by Falk, 1980a).

In conclusion, the two causative agents of MCF, AHV-1 and the agent of SA-MCF, have numerous biological properties in common with the gammaherpesviruses HVS, HVA, *H.aotus* type 2 and *H.sylvilagus*. These are the ability to cause a lymphoproliferative disease in species related to the natural host while causing only an asymptomatic infection in the natural host (but see above for exceptions). The viruses remain latent within lymphoid cells of the host, probably for the lifetime of the host, but can be reactivated in the host following immunosuppression or, *in vitro*, by culture. Lymphoblastoid cell lines possessing cytotoxic activity and

containing viral DNA sequences can be cultured from tissues derived from affected animals. The properties of these cell lines varies: only a proportion can transmit disease and some, but not all, require the growth factor IL-2.

6.1.2 Genomic organisation.

The results described in Chapters 2 and 3 of this thesis provide the first evidence that AHV-1 possesses a similar genomic organisation to that of the *H.saimiri*-like herpesviruses (herpesvirus genus *Rhadinovirus*). The overall genomic length estimated as approximately 160 kbp from restriction endonuclease analysis (Chapter 3) is of a similar order to that of this group of herpesviruses (Table 1.3.1.2). The restriction endonuclease analysis described in Chapter 2 showed the existence of tandem direct repeat sequences in the genome, while restriction mapping experiments indicated a terminal location (Chapter 3). The number and size of these repeat sequences are similar to those of HVS, HVA, *H.sylvilagus* and *H.aotus* type 2 (see Table 1.3.1.2). Finally, work completed before the start of this study demonstrated a heterogeneity in the guanine plus cytosine content of the virus, with the high G+C DNA corresponding to the repeated DNA (Bridgen *et al.*, 1989). The figures obtained (50% G+C for the whole genome, 72% G+C in the repeated DNA) correlate well with those obtained for the *H.saimiri*-like herpesviruses (Table 1.3.1.1).

The C500 isolate of WC11 possesses only a single repeated sequence of 1050 bp while WC11 possesses three major related repeated sequences of 950, 1050 and 1800 bp and numerous minor related sequences. This is similar to the organisation of *H.aotus* type 2, which possesses two major repeated sequences of 2.1 and 2.3 kbp at low passage. These are observed as sequences of 2.3 and 2.7 kbp following high passage, with 1.3 kbp DNA common to both sequences. *H.aotus* also possesses numerous related repeated sequences of intermediate size interspersed between the major sequences (Fuchs *et al.*, 1985). Cross-hybridisation and restriction

analyses have indicated a relationship between the major repeated sequences of AHV-1 (Chapter 2) while the indistinctly resolved *Mbo*I fragment ladders suggested that there is interspersion of additional minor repeated sequences between the major repeats.

6.2 Review of the genomic studies of AHV-1.

Several authors have published limited restriction AHV-1 endonuclease profiles and hybridisation results during the last six years. Ludwig (1983) described both C500 and WC11 as having molecular weights of approximately 80×10^6 (which corresponds to a genome length of 120 kbp.). He also provided restriction profiles of WC11 DNA digested with *Hin*dIII, and C500 DNA digested with *Hin*dIII and *Eco*RI. Other restriction endonuclease profiles of WC11 DNA have been provided by Osorio *et al.*, 1985 (*Bam*HI, *Bgl*II, *Eco*RI and *Hin*dIII), Seal *et al.*, 1987 (*Bam*HI and *Hin*dIII), Shih *et al.*, 1988 (*Bam*HI, *Eco*RI, *Hin*dIII and *Xba*I) and by Rossiter *et al.*, 1989 (*Bam*HI, *Eco*RI *Hin*dIII and *Pst*I). All of these profiles are consistent with each other and with those described in this thesis with the exception of the *Bam*HI profile provided by Shih *et al.*, 1988. This contains a 10 kbp fragment not observed in other profiles. Shih *et al.*, also reported the cloning of a 5.45 kbp WC11 *Bam*HI fragment, while Osorio *et al.* compared the restriction profiles of WC11 with those thought to be derived from *Connochaetes gnou* (Osorio *et al.*, 1985; Castro *et al.*, 1982).

A paper by Seal *et al.*, (1989) was published as this thesis was being completed, comparing the polypeptides, restriction endonuclease profiles and G+C content of AHV-1 and AHV-2. This paper showed caesium chloride density gradient profiles of WC11 DNA with almost identical estimates for the G+C content of WC11 total genomic and repeated DNA as those obtained by Dr A.J. Herring. The length of the WC11 genome was estimated as approximately 115 kbp from the summation of restriction endonuclease

fragments. Since the repeated DNA was not included in this calculation, nor the unique DNA at the junctions of the unique and repeated DNA, the estimate essentially confirmed the results described in this thesis.

6.3 The relationship between AHV-1 and the SA-MCF agent.

The hybridisation experiments described in Chapter 5 provide good evidence that the agent of SA-MCF is a herpesvirus closely related to AHV-1. AHV-1 and the putative SA-MCF agent present within lymphoblastoid cell lines derived from SA-MCF -affected animals (Section 5.0) showed extensive homology in the unique regions of the genome but no detectable homology in the repeated DNA. A similar situation is observed between *H.saimiri* and *H.ateles*, which show homology in 35% of the unique DNA but in only 10% of the repeated region as measured by reassociation kinetics. There is sequence divergence of approximately 9% in the cross-hybridising regions of the unique DNA (Fleckenstein *et al.*, 1978). The repeat DNA is non-coding (Bankier *et al.*, 1985) and is thought to undergo rapid evolution as a result.

The level of homology between AHV-1 and the agent of SA-MCF is extremely high. Under the hybridisation stringencies used the expected duplex melting temperature (Tm) would be approximately 68°C (Section 7.5.4), while the hybridisations were performed at 42°C, ie. 26°C below the estimated Tm. Since the Tm of a duplex is reduced by 1.4°C for each base mismatch, only hybrids with 18% or less mismatch would be thermally stable at that temperature (Hyman *et al.*, 1973; Hirai *et al.*, 1984).

Despite the propagation of a significant proportion of the genome of the agent of SA-MCF *in vitro*, this agent has not formally been classified as a virus since it has not yet been isolated completely. The genomic studies described in this thesis together with serological data (Rossiter, 1981 and 1983; Herring *et al.*, 1989 and Reid *et al.*, 1989a) provide convincing evidence that the SA-MCF is a herpesvirus

related to AHV-1 which naturally infects sheep. It should therefore be tentatively classified as a member of the *Rhadinovirus* genus of the gammaherpesvirus subfamily. The virus should also be classified as a *Caprine* herpesvirus. Two herpesviruses have already been isolated from the sub-family *Caprinae*, to which sheep and goats belong. The herpesvirus associated with sheep pulmonary adenomatosis (Mackay, 1969) has been designated *Caprine herpesvirus* 1 (Roizman, 1982), and is a gamma-like herpesvirus of only 70-80 kbp (Scott, 1984). An alphaherpesvirus of goats (Saito *et al.*, 1974; Engels *et al.*, 1987) has been variously designated *Caprine herpesvirus* 1 (Engels *et al.*, 1987) or *Caprine herpesvirus* 2 (Roizman, 1982). The SA-MCF agent should therefore provisionally be designated *Caprine herpesvirus* 3 (CHV-3).

6.4 Future work.

Several aspects of the genomic organisation of AHV-1 remain to be elucidated. One small region within the unique region of the genome (described in Section 3.2.7) and the two junction regions between the unique and repeated DNA have not been mapped rigorously. In view of the difficulty of preparing adequate quantities of viral DNA further cloning of WC11 DNA will be necessary to complete this work. It may prove advantageous to clone the entire unique region of the WC11 genome. The sizes of the two largest WC11 *Eco*RI fragments are such that they could be cloned in a cosmid vector (Maniatis *et al.*, 1982). This would only leave the two *Eco*RI fragments which span the unique-repeat DNA junctions uncloned. The junction fragment at the 3′ end of the viral genome could be cloned as a 5.4 kbp fragment in a plasmid vector, and could be isolated using H48 as a probe (see Section 3.2.5). The junction fragment at the 5′ end of the viral genome is likely to be harder to isolate. At least part of this region could be cloned into a plasmid as a *Kpn*I-*Bam*HI or as a *Kpn*I-*Sac*I fragment, using p6f to isolate the clone. If *Sac*I cuts the unique DNA 5′ to the *Eco*RI site equivalent to the 5′ end of R65, further clones

constructed using other cloning enzymes will have to be isolated using the *Kpn*I-*Bam*HI or *Kpn*I-*Sac*I clone as the probe.

The explanation for the size heterogeneity of the DNA contained within the H10/ R65 clones is unknown, but may result from the presence of small inverted repeat sequences. The origin of the 1.3 kbp supermolar fragment observed in Figure 2.1.2 is also unknown. This sequence should be cloned in order to discover its origin and nature.

The derivation of the WC11 clones and a restriction map for this isolate now allows sequence comparison between AHV-1 and other herpesvirus DNAs to be made. In the past the relatedness of different herpesviruses has been assessed by hybridisation. However, the method of choice for comparison of the herpesviruses must now be sequence analysis, because of the specificity and speed of this technique, and the large and increasing amount of sequence data available. For example, since the beginning of this study the entire sequence of EBV (Baer *et al.*, 1984), *Varicella zoster* virus (VZV) (Davison and Scott, 1986) and HSV-1 (McGeoch *et al.*, 1988) have been determined. Sequence analysis of multiple small regions of the HVS genome has shown that not only is there sequence homology with EBV, but also that the genomes are colinear (Cameron *et al.*, 1987; Gompels *et al.*, 1988 a and b). Many of the EBV genes homologous to HVS DNA encode products which have been shown to be similar to products of both VZV and HSV. The sequencing of multiple small regions of the genome was also employed to demonstrate the similarity of Marek's disease virus with the alphaherpesviruses (Buckmaster *et al.*, 1988). The derivation of two related CHV-3 clones means that a small region of this virus also can be compared with both AHV-1 and other herpesviruses. It is somewhat fortuitous that the WC11 clone used to isolate these CHV-3 clones hybridised to HVS and has been shown to possess limited sequence homology with VZV.

Several biological questions remain unanswered. Pulsed-field experiments suggested that the AHV-1 genome contained within the functionally transformed lymphoblastoid cell line BJ/465 may be integrated into the host chromosomes, but the non-transformed cell line BJ/610 has not been examined. Recent attempts to grow this cell line following storage at -70°C in DMSO have resulted in the recovery of virus in permissive cells (Reid, personal communication). The Southern blot analysis of this cell line indicated that the unique region of the viral genome was identical to that of C500 (Section 4.1). It therefore seems likely that this cell line contains episomal copies of the entire AHV-1 genome.

The most pressing problem must be the detection and isolation of the virus of SA-MCF, CHV-3. The derivation of two CHV-3 clones will provide a means of its detection. The most successful approach is likely to be the use of CHV-3 sequence data to create primers for polymerase chain reactions (pcr) (Saiki *et al.*, 1985). However, since the CHV-3 clones were derived from a bovine lymphoblastoid cell line and not from sheep, careful sequence comparison with the AHV-1 clone p6a will be required to identify conserved sequences which are suitable for pcr amplification. It may be necessary to construct several primer pairs before a combination is found which can be used for sequence amplification of CHV-3 DNA sequences in sheep or in SA-MCF--affected animals.

A possible starting material for this technique is the nasal and ocular secretions of lambs collected during the first few months of life, by analogy with the transmission of AHV-1 in wildebeest (Mushi *et al.*, 1980). Another would be lymphatic fluid and nasal secretions from beta-methasone treated sheep which are known to be CHV-3 antibody positive, since this glucocorticoid has been shown to cause reactivation of AHV-1 in the wildebeest (Rweyemamu *et al.*, 1974).

Viral neutralising antibodies to AHV-1 have also been detected in goats (Heuschele *et al.*, 1984). Similar pcr experiments to those described above could be used to determine whether there is indeed a herpesvirus related to AHV-1 in goats, and if so, whether it is equivalent to CHV-3 and is likely, therefore, to be an additional source of MCF infection.

Another major outstanding problem concerning MCF infection is the means by which the viruses of malignant catarrhal fever induce death in susceptible animals in such a short space of time, while being present at such low copy numbers. The *in situ* hybridisation experiments described in Chapter 4 confirmed that AHV-1 is present in the tissues of affected animals at only low copy numbers. A lymphokine deregulation has been proposed as a possible means of action of AHV-1 (Reid and Buxton, 1984). One approach to resolving this problem would be to assess the levels of cytokine gene mRNAs in the tissues of affected cattle, since cloned cDNAs from some of the bovine cytokine genes are now commercially available.

The genomic characterisation of WC11 now means that it is pos-sible to approach the problem of why the C500 but not the WC11 isolate of AHV-1 is pathogenic. In HVS, a region near one end of the unique region of the molecule has been implicated in oncogenicity (Desrosiers *et al.*, 1985 and 1986; Medveczky *et al.*, 1984). An open reading frame, capable of encoding a protein designated the *Herpesvirus saimiri* transformation-associated protein (STP), has recently been identified (Murthy *et al.*, 1989). It is possible that mutations in only a single gene or small region of the AHV-1 genome could account for the difference between the C500 and WC11 isolates of AHV-1. If this is the case, co-transformation experiments similar to those described by Desrosiers *et al.*, 1984 or Medveczky *et al.*, 1989b for HVS could be performed in order to determine the genomic location of this region.

The similarity of the biological properties of the causative agents of MCF and of the *H.saimiri*-like herpesviruses discussed in Section 6.1 cannot discount the possibility that these herpesviruses act by essentially similar pathogenic mechanisms. It is undoubtedly essential to compare the DNA sequences of AHV-1 and HVS in the region corresponding to the HVS STP which may well, by analogy with HVS, be located near to one end of the unique DNA of AHV-1. If the underlying mechanisms are similar, the animal models available for MCF infection should provide a useful means of studing the disease processes induced by this group of viruses.

CHAPTER 7

MATERIALS AND METHODS.

7.1 MATERIALS

7.1.1 Enzymes and Chemicals.

AnalR grade chemicals were purchased from Fisons, BDH Chemicals Ltd., Sigma, Boehringer Mannheim and M and B. Molecular biological reagents were purchased from Amersham International, Biorad, Bethesda Research Laboratories (Gibco BRL), Boehringer Mannheim, New England Biolabs (NEB) and Pharmacia. Radiochemicals were purchased from Amersham International. Deoxycytidine 5'-[α-^{32}P] triphosphate, triethylammonium salt was purchased as an aqueous solution of 10μCi ul^{-1} and 3000 Ci mmol^{-1}. Deoxyadenosine 5'-[α-^{32}P] triphosphate, triethylammonium salt was purchased at 6000 Ci mmol^{-1}. Deoxyadenosine 5'-[α-^{35}S] thiotriphosphate, triethylammonium salt was purchased as an aqueous solution of 1000Ci mmol^{-1}. Deoxy [1',2',2,8-^{3}H] adenosine 5'-triphosphate, ammonium salt was purchased as a 1:1 ethanol:water mixture of 66 Ci/mmol. Adenosine 5'[γ-^{32}P]triphosphate, triethylammonium salt was purchased as an aqueous solution of 5000 Ci mmol^{-1}.

7.1.2 Solutions.

General DNA Solutions

TE

10mM Tris-HCl pH 8.0

1mM EDTA pH 8.0

TNE

100mM NaCl

10mM Tris-HCl pH 8.0

1mM EDTA pH 8.0

TBE (agarose gels)

134mM Tris-base

44mM boric acid

2.6mM EDTA Made up as a 10x stock

TBE (sequencing gels)

100mM Tris-base

88mM boric acid

2 mM EDTA Made up as a 10x stock

TAE

40mM Tris base

20mM sodium acetate

1mM EDTA

Adjusted to pH 7.4 with CH_3COOH Made up as a 10x stock

Loening E buffer

36mM Tris base

1mM EDTA

30mM $NaH_2PO4.2H_2O$

pH should be 7.6-7.8 Made up as a 5x stock

Modified Laemmli buffer

25mM Tris base

192mM glycine Made up as a 4x stock

DNA gel loading dye

10% (w/v) Ficoll

0.05% (w/v) xylene cyanol

0.025% (w/v) bromophenol blue

Nick-translation buffer

53mM Tris-HCl pH 7.5

5.3mM $MgCl_2$

5 $\mu g\ ml^{-1}$ bovine serum albumin (BSA)

0.02mM d(GTP, ATP, TTP)

35mM β-mercaptoethanol Stored at -20°C

Oligolabelling buffer (OLB)

Solution O: 125mM $MgCl_2$
1.25M Tris-HCl pH 8.0

Solution A: 950µl solution O
18µl β-mercaptoethanol
25µl 20mM dATP
25µl 20mM dGTP
25µl 20mM dTTP
5µl 20mM dCTP (when dCTP was the labelled deoxynucleotide)

Solution B: 2M Hepes pH 6.6

Solution C: Hexadeoxyribonucleotides (Pharmacia) in 3mM Tris-HCl/ 0.2mM EDTA pH 7.0 at 90 OD units ml^{-1}

OLB was made up by mixing solutions A, B and C in the ratio 2:5:3 (initially) or, later, in the ratio A:B:C:distilled water of 20:50:6:24 and was stored in 10µl aliquots at -20°C

10x ligation buffer (ligation of cohesive ends)

660mM Tris-HCl pH 7.2
100mM $MgCl_2$
100mM β-mercaptoethanol
10mM EDTA pH 7.2
1mM ATP

10x ligation buffer (ligation of blunt ends)

500mM Tris-HCl pH 7.8
100mM $MgCl_2$
200mM Dithiothreitol (DTT)
10mM ATP

2x Bal 31 buffer

24mM $CaCl_2$
24mM $MgCl_2$
400mM NaCl
40mM Tris-HCl pH 8.0
2mM EDTA pH 8.0

DNAse 1 dilution buffer

50% (v/v) glycerol

0.05% (w/v) BSA

45mM $(NH_4)_2SO_4$

4.5mM β-mercaptoethanol

22.5mM Tris-HCl pH 7.5

Phage Packaging solutions

Buffer A

20mM Tris-HCl pH 8.0

3mM $MgCl_2$

1mM EDTA pH 7.5

7mM β-mercaptoethanol (added after sterilisation)

Buffer A was stored at -20°C

Buffer M1

6mM Tris-HCl pH 7.5

18mM $MgCl_2$

30mM Spermidine

60mM Putrescine

15mM ATP

28mM β-mercaptoethanol

DNA transfer and hybridisation solutions

Denaturation buffer

0.5M NaOH

1.5M NaCl

Neutralisation buffer

0.5M Tris-HCl

3.0M NaCl

neutralised to pH 7.4 with HCl.

20 x SSC (standard saline citrate)

3.0M NaCl

0.3M $Na_3H_2O_7$ (sodium citrate)

Ammonium acetate

1M CH_3COONH_4 (ammonium acetate)

20mM NaOH (to pH 8.0)

40x Denhardt's solution

0.8% (w/v) BSA (pentax fraction V)

0.8% (w/v) Ficoll

0.8% (w/v) polyvinylpyrrolidone, molecular weight 44,000

Salmon Sperm DNA

The DNA was made up as 5mg ml^{-1} stock, and 1.5ml aliquots were sonicated for 2 x 10 seconds.

Prehybridisation solution (filters)

50% (v/v) formamide

4 x SSC

0.1% (w/v) sodium dodecyl sulphate (SDS)

10 x Denhardt's solution

100 µg ml^{-1} sonicated salmon sperm DNA.

Hybridisation solution

As prehybridisation solution but only 1 x Denhardt's solution

Hybridisation/Prehybridisation solution

(*in situ* hybridisation)

50% (v/v) de-ionised formamide

600mM NaCl

10mM Tris-HCl pH 7.5

1mM EDTA pH 7.5

1 mg ml^{-1} BSA

0.02% (w/v) polyvinyl-pyrrolidone

0.02% (w/v) ficoll

200µg ml^{-1} sonicated salmon sperm DNA

10mM DTT

Electron microscopy solutions

Sodium carbonate solution

132mM Na_2CO_3

5mM EDTA

25% (v/v) HCHO

Denaturation solution: 0.25ml 1.0 or 1.25M NaOH added to 4ml sodium carbonate solution

Neutralisation solution: 0.25ml 0.1 dilution HCl added to 4ml sodium carboate solution

Spreading solution

5 µl DNA (at approximately 20 µg ml^{-1})

8 µl molecular weight standards (pAT153, M13)

140 µl 0.3 M Tris-HCl, 0.03M EDTA

210 µl formamide

37 µl distilled water

40 µl cytochrome C (2 mg ml^{-1})

7.1.3 Media.

All media were sterilised by autoclaving for fifteen minutes at 1.05 kg cm^{-2}. Quantities refer to 1 litre except where stated. The antibiotic ampicillin was added to 100µg ml^{-1} after autoclaving where appropriate.

Luria broth (LB)

Difco Bacto Tryptone	10g)
Difco Bacto yeast extract	5g) pH 7.2
NaCl	5g)

L agar

LB with 15g l^{-1} of Difco agar

BBL agar

Baltimore Biological Laboratories trypticase	10g
NaCl	5g
Difco agar	10g

BBL top agar

Baltimore Biological Laboratories trypticase	10g
NaCl	5g
Difco agar	6.5g

Phage buffer

KH_2PO_4	3g
Na_2HPO_4 (anhydrous)	7g
NaCl	5g
0.1M $MgSO_4$	10ml
0.01M $CaCl_2.2H_2O$	10ml
1% (w/v) gelatin solution	1ml

5x Spizizen Salts

$(NH_4)_2SO_4$	10g
K_2HPO_4	70g
KH_2PO_4	30g
$Na_3C_6H_5O_7.2H_2O$ (tri-sodium citrate)	5g
$MgSO_4.7H_2O$	1g

Minimal agar

Difco agar		6g
5 x spizizen salts		80ml
20% (w/v) glucose		4ml
2mg ml^{-1} vitamin B1		0.1ml
Distilled water	to	400ml

The spizizen salts, glucose and vitamin B1 were added after autoclaving.

YEP medium

20g glucose
20g bacto-peptone
10g yeast extract
0.04g adenine
0.04g uracil

PBS

150mM NaCl
6mM K_2HPO_4
4mM KH_2PO_4 pH should be 7.0

Chapter 7

7.1.4 Bacterial and yeast strains.

Strain	Genotype	Reference/Source
NM522	*hsd* (M,S) Δ*lac*, Δ*pro*, *supE*, *thi*, F' *pro* $A^{+}\beta^{+}$ *lacI*^{q}, *lacZ* ΔM15, *traD*36	Gough and Murray (1983)
C600	*supE*44, *thr*1, *leuB*6, *lacY*1, *thi*1, *tonA*21, λ-	Appleyard (1954)
NM533 =BNN102	*hfl*, *hsdR* C600 derivative	Young and Davis (1983)
NM621	*hsdR*, *recD*, *mcrA*, *mcrB* C600 drivative	Whittaker *et al.* (1988)
5K	*hsdR*, *trpR* C600 derivative	Hubacek and Glover (1970)
NM534	= ED8689 (P2 cox 3)	Prof N.E.Murray
ED8654	*supE*, *supF*, *hsdR*, *lacY*, *met*, *trpR*	Murray *et al.* (1977)
NM531	*recA*13 ED8654 derivative	Prof N.E.Murray
NM514	*hflA*, *hsdR*, *mcrA*	Murray (1983a)
BHB2688	N205 *rec A* (*imm*^{434},c*Its*, b2, *Eam*4, *Sam*7)/λ	Hohn (1979)
BHB2690	N205 *rec A* (*imm*^{434},*cIts*, b2,*red*3,*Dam*15 *Sam*7)/λ	Hohn (1979)
JM101	*supE*, *thi*, Δ(*lac proAB*) {F', *traD*36, *proAB*, *lacI*^{q}, *lacZ*, ΔM15}	Yanisch-Perron *et al.* (1985)
MC1061	*araD*139, Δ(*ara*, *leu*)7697 Δ*lacX*74, *galU*, *galK*, *hsdR*, *strA*	Casadaban and Cohen (1980)
YPH148	(yeast)	Rimm *et al.*, 1988

7.1.5 Cloning Vectors.

7.1.5.a Phage vectors.

Phage	Vector type	Reference
λNM1149	Lambda insertion vector	Murray (1983a)
λEMBL4	Lambda replacement vector	Frischauf *et al.* (1983)
λEMBL3	Lambda replacement vector	" "
M13mp18	*E coli* bacteriophage M13-based vectors, *lac*Z gene	Norrander *et al.* (1983)

7.1.5.1 Plasmid vector.

pUC13 Messing (1983)

pUC13 conferred ampicillin resistance to the host strain.

METHODS

7.2 General Methods.

Unless specified to the contrary, all reactions in this and the following sections were performed at room temperature.

7.2.1 Quantitation of DNA.

The concentration of DNA preparations was assessed by a measurement of the OD_{260} using a spectrophotometer. An $O.D_{260}$ = 1 corresponds to 50μg ml^{-1} DNA. DNA preparations free from protein possessed an OD_{260}:OD_{280} ratio approaching 2.0.

7.2.2 Preparation of dialysis tubing.

Dialysis tubing was cut to 50-70cm lengths, rinsed with distilled water and boiled for 10 minutes in 2mM EDTA pH 7.5. Tubing was stored in 50% (v/v) ethanol, 1mM EDTA pH 7.5 at 4°C, and rinsed with distilled water immediately prior to use.

7.2.3 De-ionisation of formamide (Maniatis *et al.*, 1982).

Formamide for use in *in situ* hybridisation experiments only was de-ionised using Biorad Bio-rex MSZ 501 or AG 501-X8 mixed bed resin. 50 ml formamide was de-ionised with 5g resin for 1 hour, the resin removed by filtration through 3MM paper and the de-ionised formamide stored at -20°C.

7.2.4 Gel photography.

Uv-illuminated ethidium bromide-stained gels were photographed using Ilford HP5 film at F4.8. Films were exposed for 20 seconds through a red filter, developed for 5 minutes in Ilford microphen, development stopped in 3% (v/v) acetic acid (30 seconds) and the films fixed in Ilford hypem for 4 minutes. Films were washed well before drying. Photographs at the Moredun Research Institute (MRI) were taken using a Polaroid camera set at F8 for 0.5 second using Polaroid 667 positive film.

7.2.5 Autoradiography.

Autoradiography of ^{32}P-labelled DNA was performed using Cronex 4 X-ray film. Films were pre-flashed and exposed at -70°C using an intensifying screen, and developed in an Agfa X 1 automatic film processor. The Fuji films used at MRI were developed using Photosol Ltd CF40 and CD18 chemicals.

7.2.6 Caesium chloride density centrifugation.

(See also 7.7.7 and 7.7.15). Caesium chloride (CsCl) density gradient centrifugation of AHV-1 DNA was performed as follows. 5ml gradients with an initial density of 1.72 g ml^{-1} (57% w/v in CsCl) were formed in TE buffer pH 7.5. The OD_{260} profile was measured by upward displacement of the gradient using an "ISCO" model 184 density gradient fractionator with a uv monitor. Fractions of 0.2 ml were collected and the density determined by refractometry.

7.2.7 Spectrophotometry.

Estimates of the molar ratios of DNA fragments separated by agarose or polyacrylamide gel-electrophoresis were derived by scanning autoradiograms and silver-stained polyacrylamide gels using a Joyce-Loebl spectrophotometer. Silver-staining of DNA in polyacrylamide gels is known to occur in a stoichiometric fashion (Whitton *et al.*, 1983).

Estimates of the areas of peaks produced by the spectrophotometric scanning of gels were made from the mass of replicas of the peaks photocopied and cut out from thick paper. The bases of the peaks were defined by drawing a line between the minima of the curves to either side of the peaks.

7.3 Digestion and modification of DNA.

7.3.1 Precipitation of nucleic acids with ethanol (Maniatis *et al.*, 1982).

DNA was precipitated from aqueous solution by addition of 0.1 volume 3M sodium acetate pH 5.0 and 2-3 volumes of cold absolute ethanol, and incubation at -70°C for 20-120 minutes depending on the volume. DNA precipitated from solution was sedimented by centrifugation in a microfuge for 5 minutes or in the HB-4 rotor of a Sorvall centrifuge at 10 krpm for 10 minutes at 4°C. DNA pellets were rinsed in 70% ethanol, dried in a vacuum desiccator and dissolved in TE. For precipitations of nanogram amounts of DNA, 20µg ml^{-1} of mussel glycogen (Boehringer) was added as carrier; this did not affect subsequent digestion by endonucleases.

7.3.2 Precipitation of DNA with isopropanol (Maniatis *et al.*,1982)

Isopropanol precipitation was used for selective precipitation of DNA molecules of greater than approximately 200 bp. 0.4 volume of ammonium acetate pH 4.8 and 1 volume isopropanol was added to the DNA solution, which was incubated for 10 minutes. DNA was sedimented as described for ethanol precipitation.

7.3.3 Restriction endonuclease digestion of DNA.

Restriction endonuclease buffers were used as recommended by the manufacturers. Digestions were normally performed in 20µl, using 3 units enzyme per µg DNA for 2-16h at 37°C or 30°C (*Sma*I). Enzymes were heat inactivated where possible at 70°C for 10 minutes prior to the ligation of restriction fragments.

7.3.4 Bal 31 digestion (Maniatis *et al.*, 1982).

Bal 31 is a Ca^{2+}-dependent exonuclease which degrades both the 3' and 5' strands of double-stranded DNA. The activity of *Bal* 31 was assayed by digestion of λcI857 *Hin*dIII restriction fragments. 5µg DNA in 25µl was added to 25µl 2 x Bal 31 buffer. 10µl samples were taken before addition of the exonuclease and after 1, 2 and 5 minutes digestion at 25°C. Digestion was stopped by phenol extraction. The samples were vortexed with 40µl TE and 50µl phenol, and the aqueous and phenol phases separated by centrifugation for 30 seconds in a microfuge. The aqueous phases were loaded directly onto an agarose gel. The molecular weights of the digested fragments were estimated and used to calculate the activity of the *Bal* 31.

7.3.5 Ligation of DNA (Murray *et al*, 1977; Maniatis *et al.*, 1982).

Ligation of DNA molecules was performed in 10-50µl for 3-18h at 16°C, generally using a 3:1 molar ratio of insert:vector DNA. DNA molecules with complementary cohesive ends were ligated at a concentration of 20-100µg ml^{-1} using one-tenth volume of 10x ligation buffer (cohesive ends) and 1 units T4 DNA ligase (BCL). DNA molecules with blunt ends were ligated at a concentration of 100-500µg ml^{-1} using one-tenth volume of 10x ligation buffer (blunt ends) and 20 units T4 DNA ligase (BCL).

7.4 Electrophorectic and associated techniques.

7.4.1 Size separation of DNA fragments by agarose gel electrophoresis.

DNA fragments of 200bp to 25kbp were separated by agarose gel-electrophoresis through 0.3-2% (w/v) agarose in TBE

buffer in BRL or Pharmacia submerged gel tanks. Digests were loaded into the sample wells with 0.25 volume DNA gel loading dye, and electrophoresed at 2-8V cm^{-1}. λcI857 *Hin*dIII or *Hin*dIII/*Eco*RI fragments, or the BRL 1kbp ladder, were used as molecular weight standards. DNA was visualised on a uv transilluminator (UV Products) at 254nm following staining with 10μg ml^{-1} ethidium bromide.

7.4.2 Pulsed-field gel-electrophoresis.

Initial separation of large (>20 kbp) DNA fragments was achieved by the orthogonal-field-alteration-gel-electrophoresis (OFAGE) apparatus first described by Carle and Olsen (1984). Fragments were separated with a pulse time of 10 seconds and a voltage gradient of 20V cm^{-1}. Later experiments involved use of a "Waltzer" apparatus (Southern *et al.*, 1987), which was built at the MRC unit of the Western General Hospital, Edinburgh. In this apparatus, samples loaded on a circular gel were moved with respect to a constant electric field (Fig. 7.4.2.1 below).

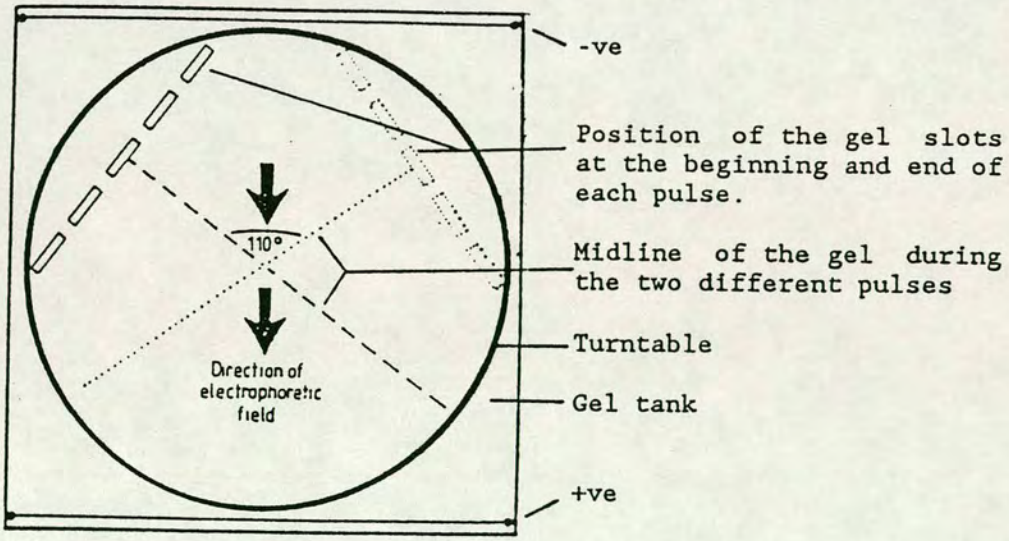


Figure 7.4.2.1
Modified from Southern *et al.*, 1987

DNA samples were either applied directly to the wells or were embedded into agarose blocks which were placed in the wells. DNA fragments were separated through voltage gradients of 10-20V cm^{-1} with pulse intervals of 25-70 seconds. The apparatus achieved good separation of fragments of 100-1000kbp. DNA markers used with this apparatus were ligated lambda molecules (50-400kbp) and whole yeast chromosomes (92-1,580 kbp).

7.4.2.1 Embedding of genomic DNA into agarose plugs.

Experiments to determine the cellular location of viral DNA within lymphoblastoid cell-lines derived from MCF -affected animals necessitated the embedding of viable cells in agarose and lysis of the cells in these blocks in order to avoid shearing the DNA. The method employed was that of Van den Berg *et al.* (1988).

Cultured lymphoblastoid cells were pelleted by centrifugation at 2krpm in a bench centrifuge for 5 minutes. The cell pellet was immediately resuspended in Tris-EDTA buffer (10mM Tris-HCl pH 7.5, 10mM EDTA) to a concentration of 5 10^7 cells ml^{-1}, and warmed to 37°C. An equal volume of 2% low melting-point (lmp) agarose (in Tris-EDTA buffer) at 37°C was mixed with the cell suspension and 150µl aliquots pipetted into the wells of a microtitre plate and allowed to set. The agarose plugs were incubated in SE buffer (0.5M EDTA pH 8.0, 1% SDS) for 1 hour. This buffer was replaced with additional SE buffer supplemented with 500µg ml^{-1} proteinase K, and the plugs incubated at 37°C for 12-18 hours. The plugs were rinsed three times in SE buffer over a period of 1-6 hours, and then stored in SE buffer at 4°C. Plugs were rinsed in TE prior to gel electrophoresis.

7.4.2.2 Preparation of yeast chromosomes.

Yeast chromosomes were prepared according to an unpublished method of Mr J. Maule of the MRC unit of Western General Hospital, Edinburgh. 100ml YPH148 cells (gift of Dr H. Lehrach) were grown to mid-log phase at 30°C

in YEP medium (Olson *et al.*, 1979) and harvested by centrifugation at 1800 rpm in an MSE Mistral 6 for 10 minutes. The pellet was washed twice in ice-cold 50mM EDTA pH 7.5, and resuspended in 3ml of the same solution. 5ml of 1% lmp agarose (prepared in 0.125M EDTA pH 7.5) at 42°C was mixed with the cell suspension, and 1ml SCE, 50µl β-mercaptoethanol and 15mg Boehringer protoplast-forming enzyme were added. (SCE = 1M sorbitol, 0.1M trisodium citrate, 0.06M EDTA pH 7.0). (Protoplast-forming enzyme destroys β-1-3 glycan linkages). 150µl aliquots were pipetted into the wells of a microtitre plate and allowed to set. The plugs were incubated for 12-18 hours at 37°C in 0.45M EDTA, 10mM Tris-HCl pH 8.0, 7.5% (v/v) β-mercaptoethanol. This solution was replaced by a similar solution containing the additional components of 1% (w/v) SDS and 1mg ml^{-1} proteinase K, and incubation was performed for 6 hours at 50°C. The plugs were stored in 0.45M EDTA, 10mM Tris-HCl pH 8.0, 7.5% (v/v) β-mercaptoethanol and 1% (w/v) SDS at 4°C and rinsed in TE prior to agarose gel electrophoresis.

During early preparation of yeast cell plugs, aliquots of yeast cells were treated with protoplast forming enzyme in the absence of agarose to allow visualisation of protoplast formation by light microscopy. Water drawn onto the microscope slide immediately lysed yeast protoplasts, but not yeast cells with intact cell walls.

7.4.3 Gardella gels (Gardella *et al.*, 1984).

Gardella gels were also used to determine the location of viral sequences within lymphoblastoid cell lines. The method was performed exactly as described by Gardella *et al.* Essentially the method involves the electrophoresis of DNA released from viable cells at the initiation of electrophoresis by SDS and proteinase K contained within the agarose gel. Following electrophoresis, the DNA was transferred to a nylon membrane and hybridised with an appropriate probe. The method depends on the different mobilities of covalently closed circles and linear DNA.

7.4.4 Isolation of DNA from agarose gels.

DNA fragments were purified from low melting-point agarose following excision as follows. Equal volumes of the excised fragment and TBE/0.2M NaCl were added to an Eppendorf tube. The agarose was melted at 60°C for 10 minutes and removed by two phenol extractions using 0.75 volume of phenol pre-equilibrated in TBE/0.1M NaCl. The aqueous and phenol phases were separated by centrifugation for 15 minutes in a microfuge. DNA was concentrated by addition of 4 volumes of butan-1-ol; DNA in the aqueous phase was precipitated by two ethanol precipitations.

Gene clean (Stratech) was employed in the isolation of DNA fragments from TAE agarose gels. The yield was approximately 70% using both methods.

7.4.5 Transfer of DNA from agarose gels to nylon or nitrocellulose membranes.

DNA was transferred from agarose gels to nitrocellulose (Sleicher and Schüell) or nylon (Amersham Hybond-N) membranes by the modification of the method of Southern *et al.* (1975) described by Smith and Summers (1980). Gels were incubated in 0.25N HCl for 15 minutes to depurinate the DNA, rinsed in distilled water and incubated in 0.5M NaOH, 1.5M NaCl for 30 minutes to denature the DNA, then in the neutralising transfer medium, ammonium acetate, for 60 minutes. Bi-directional blots were arranged by placing one sheet of nitrocellulose/nylon then two sheets of blotting paper soaked in ammonium acetate above and below the gel, and placing a 3cm layer of blotting paper cut to size above and below the sandwiched gel. A weighted glass plate was placed on the gel, and transfer performed at room temperature for 2-16 hours. The filters were rinsed in 2 x SSC and DNA fixed to the membrane by 5 minutes uv - fixation (Hybond-N) or by baking for 90 minutes under vacuum (nitrocellulose).

7.4.6 Size-fractionation of DNA fragments by polyacrylamide gel -electrophoresis (McClenaghan *et al.*,1984).

Polyacrylamide gel-electrophoresis was used to resolve DNA fragments of 0-2000bp. A 30% (w/v) acrylamide/1% (w/v) bisacrylamide stock solution was prepared, filtered and stored in the dark at 4°C. 60ml 7.5% acrylamide/0.25% bisacrylamide in Loening E buffer (Loening, 1969) was de-aerated and mixed with 30µl TEMED and 300 µl freshly prepared 10% (w/v) ammonium persulphate and used to prepare a vertical gel using a home-made gel apparatus. Gels were left for at least two hours to set. The samples were loaded with 0.5 volume agarose gel DNA loading dye containing 10% (w/v) sucrose. Electrophoresis was carried out at 5V cm^{-1} for 18 hours.

7.5% (w/v) discontinuous gels with a 3% (w/v) acrylamide stacking gel were used to give better resolution of 2-4 kbp fragments. The gels were prepared with Laemmli buffer (Laemmli, 1970) without SDS. The 7.5% (w/v) gel was poured, overlayed with distilled water to give a straight top to the gel, allowed to set, and a 3% (w/v) stacking gel poured. Samples were loaded and electrophoresed as described for continuous acrylamide gels. Following electrophoresis, the gels were stained with ethidium bromide or silver nitrate.

7.4.7 Silver-staining of polyacrylamide gels.

The method used was modified from that of Herring *et al.* (1982). The acrylamide gels were soaked in 10% (v/v) ethanol, 0.5% acetic acid for 20 minutes (continuous gel) or 40 minutes (discontinuous gel) with gentle shaking. Gels were stained with 11.2mM silver nitrate for 20 minutes, rinsed in distilled water, and soaked in 0.75M NaOH, 0.25% (v/v) formaldehyde for 10 minutes during which time the DNA bands became visible. Staining was enhanced by incubation in 73mM sodium carbonate solution for 30 minutes.

7.4.8 Electrophoretic transfer of DNA from polyacrylamide gels to nylon membranes.

Nylon membranes were used for electrophoretic transfer because of the higher affinity of nylon than nitrocellulose for small molecules.

Transfer was performed according to the Bio-Rad protocol. Following electrophoresis, the gel was soaked in 0.2M NaOH, 0.5M NaCl for 30 minutes, then neutralised by three 10 minute washes in TAE. A zeta-probe membrane, pre-soaked in TAE for 5 minutes, was placed on the gel. The gel was then sandwiched between two sheets of blotting paper soaked in TAE in the Bio-Rad Transblot electrophoretic transfer system. Transfer was performed at 4°C using a heat exchanger for 30 minutes at 2V cm^{-1} then 2 hours at 5V cm^{-1}.

7.5 DNA labelling and hybridisation techniques.

7.5.1 Nick-translation.

The method was based on Rigby *et al.* (1977). 0.2-0.5µg DNA in 0-4µl was added to 20µl nick-translation buffer, and 1µl 2.10^{-5}mg ml^{-1} DNAseI, 1 unit DNA polymerase I and 10-40µCi [α-^{32}P]-dCTP added. Following a 1 hour incubation at 12°C, 0.5µl reaction was removed and assayed for incorporation of [α-^{32}P]-dCTP into the DNA. The reaction was terminated and unincorporated nucleotides removed by passage through a 15cm G50 Sephadex column (Sephadex was from Pharmacia).

7.5.2 Measurement of incorporation of [α-^{32}P]-dCTP into DNA.

The percentage incorporation of [α-^{32}P]-dCTP into DNA was assayed by measurement of acid precipitable counts. 0.5µl of the nick-translation reaction was mixed with 200µl sonicated 50µg ml^{-1} calf thymus or salmon sperm DNA in a 3ml glass tube. 5µl was spotted onto a Whatman GF/C glass fibre filter and dried, while 2.5ml cold 5% (w/v) trichloroacetic acid (TCA) was added to the remainder. After 10 minutes incubation on ice, the TCA was filtered through a second filter, which was washed with further TCA and ethanol. The

dry filters were counted in 2.5ml scintillant (0.6% w/v butyl-PBD in toluene) and the figures for total and acid precipitable counts compared.

7.5.3 Primer-extension labelling of DNA.

This method was described by Feinberg and Vogelstein (1983 and 1984) and involves the extension of random hexanucleotides bound to single-stranded DNA. 50ng DNA in 35µl distilled water was boiled for 10 minutes then cooled to 37°C before adding 10µl oligolabelling buffer, 2µl of 10mg ml^{-1} BSA, 5 units Klenow and 20-50 µCi [α-^{32}P]-dCTP. The reaction was performed at room temperature for 6-18 hours. Termination and removal of unincorporated dCTP was achieved by passage of the reaction mixture through a Sephadex G50 column. Fragments isolated from low melting-point agarose gels were diluted 3 fold in distilled water and boiled prior to primer-extension labelling at 30°C.

This labelling method was used for all the *in situ* hybridisation experiments using ^{32}P, ^{35}S and ^{3}H -labelled deoxynucleotides.

7.5.3.1 DNA labelling with digoxygenin-dUTP by primer extension.

Towards the end of this study Boehringer Mannheim produced a non-radioactive labelling and hybridisation kit based on digoxygenin-dUTP. The incorporated label bound phosphatase-conjugated sheep polyclonal anti-digoxygenin, and was visualised by a coupled reaction involving the oxidation of BCIP (5-bromo-4-chloro-3-indolyl phosphate) and reduction of NBT (nitroblue tetrazolium) to coloured products which was initiated by the phosphatase.

The reactions were performed as described by the manufacturers with the exception of the development step, which was performed at 37°C.

7.5.4 Hybridisation of radioactive probes to DNA bound to membranes.

Nitrocellulose or nylon membranes were pre-hybridised in pre-hybridisation solution for at least 30 minutes with gentle agitation. Hybridisations were performed overnight in hybridisation solution using 10^4-10^5 dpm cm^{-2} probe, which was boiled for 5 minutes prior to hybridisation. Unbound probe was removed from the membranes by two 2 hour washes in 4 x SSC, 50% (v/v) formamide and 1 x Denhardt's solution at the hybridisation temperature followed by 30 minute washes in 1 x SSC then 0.5 x SSC at the same temperature. Filters were dried or placed in plastic bags before autoradiography. Initial hybridisation experiments were performed in plastic bags on a shaker at 37°C; later experiments were performed in small volumes in roller bottles on Gallenkamp rollers in a 42°C incubator.

When necessary, the melting temperature of two DNAs being hybridised (Tm) was estimated using the formula:

$$Tm = 81.5°C + 16.6 \log_{10}M + 0.41 (\%G+C) - (500/n) - 0.61 (\%formamide)$$

where n is the length of the shortest chain in the duplex
M is the salt concentration (mol per litre)
and G+C is the percent of guanine plus cytosine residues (Meinkoth and Wahl, 1984).

7.5.5 Restriction mapping of phage DNA by partial digestion and cos-end labelling (Rackwitz *et al.*, 1984).

Oligonucleotides complementary to the right cohesive end of lambda molecules (5' GGGCGGCGACCT 3') were synthesised at the AFRC Institute of Animal Physiology, Babraham. These were end-labelled using [α-^{32}P]-dATP and polynucleotide kinase.

A number of lambda partial digests were obtained by the use of varying enzyme concentrations and digestion times. Digestion was stopped by addition of EDTA pH 7.5 to 20mM, placing the digestion tube on ice and phenol extraction. The

extent of digestion was assessed by agarose gel-electrophoresis of small samples of the digests. Appropriate digests were pooled for cos-end labelling.

1µl of the labelled oligonucleotide was added to 100µl TE and 25µl 1M NaCl (200mM final concentration) and 5µl of this solution was added to 300-500ng of the partially digested lambda recombinant in a total volume of 10µl. Samples were denatured at 70°C for 3 minutes then annealed at 42°C for 30-60 minutes. DNA loading buffer was added and the samples immediately loaded onto a 0.5% agarose gel. Electrophoresis was performed overnight and the gel was photographed and dried onto Whatman DE-81 cellulose paper. The sizes of the the partial digestion fragments visualised by autoradiograp-hy were estimated and used in the construction of a restriction map.

7.6 In situ hybridisation techniques.

7.6.1 Preparation of slides.

Microscope slides were rinsed in ethanol, dipped in 1 x Denhardt's solution in 3 x SSC, rinsed in distilled water, incubated in a 3:1 mixture of ethanol:acetic acid and air dried. Slides used for tissue sections were coated in silane by the method of Pooijen-Knegt *et al.* (1982). They were incubated for 16 hours in a 2% (v/v) solution of 3-amino-propyl-triethoxy-silane (Aldrich Europe/ Beerse, Belgium) in anhydrous acetone then rinsed in acetone and distilled water. The slides were stored in an aqueous solution of 0.02% (w/v) sodium nitrate for up to 6 weeks and rinsed in distilled water immediately prior to use. A 0.01% (w/v) aqueous solution of poly-L-lysine was spotted onto the slides before use. Coverslips were cleaned in 1M HCl for 30 minutes, rinsed in distilled water, immersed in 95% ethanol for 30 minutes and dried on material gauze. They were coated in silicone by immersion in "Sigmacote", dried, baked at 180°C for 2 hours and stored at room temperature.

7.6.2 Preparation of cytospins and tissue sections.

Rabbits affected with MCF were euthanased with a mixture of carbon dioxide and fluothane (Halothane or 2-bromo-2 -chloro-1,1,1-trifluoroethane, Coopers Animal Health). Blood was collected by cardiac puncture. The anti-coagulant heparin sodium BP (Evans) was addded to 1 unit ml^{-1}. Peripheral blood lyphocytes were separated by centrifugation through Lymphoprep (Nycomed).

Tissues from MCF-affected animals were fixed in 3:1 ethanol:acetic acid for 2 hours and incubated in 70% ethanol overnight at 4°C. The tissues were dehydrated by sequential 1 hour incubations in 70%, 80% (one rinse in each), 95% and 100% (two rinses in each) absolute ethanol. They were then incubated twice for 1 hour in xylene and three times for 20 minutes in molten polywax, and then embedded in polywax. 4μm or 6μm sections were cut from the wax-embedded tissues, placed on the amino-propyl-triethoxy-silanated slides and dried at 37°C overnight. Sections were de-waxed by two 5 minute rinses in xylene, and were given two 5 minute rinses in ethanol. (Haase, 1984). Lymph node suspensions were prepared as described in 7.9.4. 10 ml of approximately 10^8 cells were cultured in plastic flasks in Iscoves medium (Gibco) supplememted with 10% (w/v) foetal bovine serum (FBS) with or without 5-10 units ml^{-1} recombinant Interleukin-2 (IL-2, kindly supplied by Biogen, Geneva). Cytospins were prepared at intervals during culture.

Cytospins were prepared from AHV-1 -infected bovine thyroid cells between 1.5 and 3 days pi. Cytospins were prepared using a Shandon Cytospin 2. 4×10^5 cells in 0.2 ml were centrifuged onto the prepared slides at 1500 rpm for 5 minutes.

7.6.3 Treatment of cells prior to hybridisation (Haase *et al.*, 1984).

Cytospins and tissue sections on microscope slides were immersed in 0.2N HCl for 20 minutes, rinsed in distilled

water, immersed in 2 x SSC at 70°C for 30 minutes, and again rinsed in water. Permeabilisation of the cells was achieved by incubation in 25mM Tris-HCl pH 7.4, 2mM $CaCl_2$, 1µg ml^{-1} proteinase K for 15 minutes at 37°C. Following two 5 minute rinses in distilled water, cells were dehydrated in two 5 minute rinses in 70% then 95% ethanol. Cellular RNA was digested with 100µg ml^{-1} RNase A in 2 x SSC for 30 minutes at 37°C in a humidity chamber. Cells were rinsed twice in 2 x SSC for 5 minutes and post-fixed in 5% (w/v) paraformaldehyde (in 2 x SSC) for 2 hours. Cells were again rinsed twice in 2 x SSC for 5 minutes, then genomic DNA was denatured by incubation in 95% (v/v) deionised formamide in 0.1 x SSC for 15 minutes at 65°C. Cells were cooled rapidly by immersion in ice-cold 0.1 x SSC for 2 minutes, and dehydrated in 70% and 95% absolute ethanol. The slides were stored in 95% ethanol for up to 2 days, until immediately before hybridisation.

7.6.4 Hybridisation and washing.

DNA probes were labelled with ^{32}P, ^{35}S or ^{3}H -dATP by the random primer-extension method of Feinberg and Vogelstein (1983 and 1984), and the activity of the probes measured by scintillation counting using ES299 scintillant. The probes were boiled for 5 minutes prior to use. The slides were taken from the 95% ethanol solution, air dried, and immediately 20µl of pre-hybridisation solution was added per slide (Section 7.1.2). Sections and cytospins were incubated at 42°C for approximately 1 hour, the pre-hybridisation solution removed, and 7µl of probe (1-5 10^5 cpm) in hybridisation solution was added (Brahic and Haase, 1978). Coverslips were placed over the cells, which were incubated overnight at 42°C. This temperature was approximately 25°C below the estimated melting temperature (Tm) of the probe-target duplex, where the Tm was calculated as follows:

$$Tm = 86°C + 10.16(\log_{10}[Na+]) - 0.56(\%\text{formamide}) + 0.27(\%\ G+C) - 877 / (\text{length, bp.})$$

(Personal communication, S. Legon).

Thus for M30 (72% G+C) used in hybridisation experiments in 600mM NaCl the calculated Tm is approximately 72°C and the optimal hybridisation temperature is 47°C.

Slides were rinsed in 4 x SSC for two x 5 minutes to remove most of the unbound probe and were then washed in 600mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0 and 50% formamide for two x 1 hour at 42°C, and 2 x SSC for two x 5 minutes. The slides were then dehydrated by 5 minute rinses in 300mM ammonium acetate/70% ethanol then in 300mM ammonium acetate/95% ethanol.

7.6.5 Autoradiography.

Slides were dipped in Kodak K2 emulsion in complete darkness, allowed to dry, and stored in the dark for up to 2 weeks (^{32}P-labelled probe) or up to 13 weeks (^{3}H-labelled probe).

7.6.6 Assessment of the in situ hybridisation results.

In situ hybridisation slides were viewed, using a Reichert microscope, by projection of the image onto a 15 cm diameter screen. The number of cells positive for viral DNA were assessed by scanning the entire slide under high power and oil immersion. Estimates of the total number of cells were derived for some sections and cytospins under low magnification. A 2cm grid was drawn onto the screen and the mean number of cells per 2cm square of 10 squares multiplied by the number of squares covering the section/cytospin gave an estimate of cell number. The cytospins were found to all have between $1\ 10^5$ and $5\ 10^5$ cells per slide. The grain density per virus-infected cell was found to be significantly greater than that for uninfected cells, usually 50-100 fold higher, therefore grain densities were not counted for each slide.

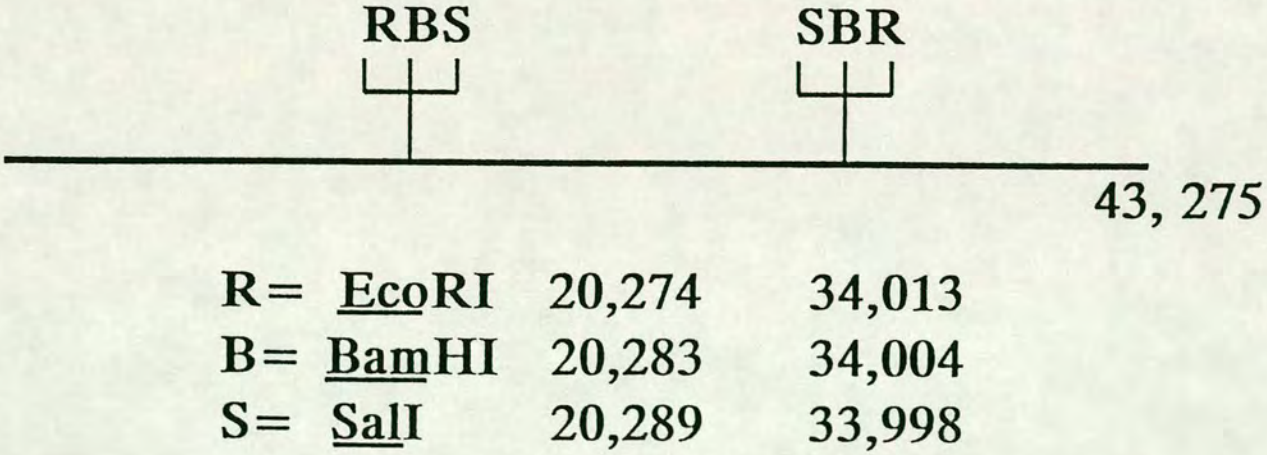
7.7 Bacteriophage and Plasmid Techniques.

The λNM1149 and λEMBL cloning vectors.

The λNM1149 and λEMBL vectors were chosen for the cloning work because of their large insert capacity (0-9 and 9-22 kbp respectively) and the ease of selection of recombinant molecules on the appropriate host strain.

λNM1149 is an insertion vector which possesses unique *Hin*dIII and *Eco*RI sites at 28,661 and 28,897 bp respectively (Murray, 1983b). Both these sites lie in the cI (repessor) gene, and insertion of foreign DNA at these sites usually leads to inactivation of the lambda repessor and consequent inability of the phage to lysogenise. Recombinant phage can be selected on *hfl*- host strains, for example NM514 or NM533, which only permit lytic phage growth. The small size of the parent phage (39,713 bp) means that recombinant phage grow more vigorously than the parent; they also have a clear rather than a turbid plaque morphology (Murray, 1983a and b).

λEMBL4 is a replacement vector with cloning sites as shown below (Figure 7.7.1). In λEMBL3, the restriction endonuclease sites at the multiple cloning regions are present in the reverse order (Murray, 1983b).



Digestion of λEMBL4 with *Eco*RI isolated the central fragment of 14 kbp which contains the non-essential *red* and *gam* genes (Kaiser and Murray, 1985). Further digestion with *Bam*HI, followed by isopropanol digestion to remove the small *Bam*HI/ *Eco*RI fragments, prevented religation of this central fragment to the vector arms. The vector arms were ligated to *Eco*RI-digested insert DNA and the phage DNA packaged. Phage were plated using C600 cells or the *hsd*R derivative, 5K. Spot tests were made using the strains NM531 and NM534; only recombinant phage grew on the P2-lysogenic strain NM534, which only allows the growth of phage deficient in the *gam* gene.

7.7.2 λ plate lysates.

Single, well isolated plaques were picked into 0.5ml phage buffer containing a drop of chloroform and vortexed to mix. 5µl and 50µl of the diluted phage (ideally 10^5 plaque forming units, pfu) were added to 0.1ml phage buffer, and adsorbed to 0.1ml of a fresh overnight culture of cells supplemented with 10mm $MgSO_4$ for 10 minutes. Cells were plated onto fresh, thick L plates in 2.5ml molten BBL top agar supplemented with 10mM $MgSO_4$. The cells were incubated at 37°C until confluent lysis had occurred (6-8 hours for λNM1149 or overnight for recombination deficient *red gam* phage). The plates were overlayed with 4ml of L broth at 4°C for 6-16 hours. The lysates were collected and transferred to Bijoux bottles; cells and debris were removed following pelleting in a bench centrifuge (2000 krpm for 5 minutes) and the phage stocks stored at 4°C after addition of 5 drops of chloroform.

7.7.3 Phage titration.

Serial dilutions of the phage stock were made in phage buffer. 0.1ml of 10^{-6} and 10^{-8} dilutions were mixed with 0.1ml of a fresh overnight culture of cells supplemented with 10mM $MgSO_4$, adsorbed for 10 minutes, and plated out on dry BBL plates in 2.5ml molten 10mM, $MgSO_4$-supplemented BBL top agar. Controls were always included to ensure that

there was no phage contamination of the cells or buffer. Plates were inverted and incubated overnight at 37°C. The phage titre (pfu ml^{-1}) was calculated from the number of plaques per plate.

7.7.4 Phage liquid lysates.

The bacterial strains used for growing up phage were C600 or ED8654 for λNM1149 and its derivatives, while NM621 was used for *red gam* phage.

A fresh overnight culture of cells was diluted 1:100 into L broth supplemented with 10mM $MgSO_4$, and incubated with vigorous shaking until the OD_{650} reached 0.5 (corresponding to 2.10^8 cells ml^{-1}). Phage were added to a multiplicity of infection (moi) of 0.1-1.0, and the incubation continued. The OD_{650} generally rose to above 2.0, then fell on cell lysis (2-4h after the addition of phage). When the OD_{650} reached a minimum, chloroform (0.5ml $500ml^{-1}$) was added, and the flasks were shaken for a further 10 minutes. The lysates were clarified by centrifugation at 2000 krpm in a bench centrifuge, then titred.

7.7.5 Phage spot tests

Serial dilutions of the phage stock were made in phage buffer. A bacterial lawn was prepared by plating 0.1ml of a fresh overnight culture of cells in 2.5ml 10mM $MgSO_4$ -supplemented BBL top agar onto a BBL plate. 5ul aliquots of 10^{-4}, 10^{-6} and 10^{-8} dilutions of the phage were spotted onto the lawn, and the cells incubated overnight at 37°C. Spot tests were used as a quick method of titering phage and to determine the presence of an insert in EMBL vectors.

7.7.6 Small-scale preparation of phage DNA.

Numerous methods were employed, using phage plate lysates grown on agarose plates or liquid lysates, but the method described below, based on Maniatis *et al.*, 1982), gave the most reliable results.

100µl of a fresh overnight culture of cells was mixed with 10^8-10^9 phage in an Eppendorf tube and allowed to adsorb for 20 minutes. The cells and phage were transferred to a sterile Bijoux bottle containing 4ml L-broth supplemented with 10mM $MgSO_4$ and the cells were shaken at 37°C until lysis occurred (3-5h). A drop of chloroform was added, the cells were shaken for a further 5 minutes and then pelleted at 2krpm for 5 minutes in a bench centrifuge. The supernatant was transferred to a fresh Bijoux, 5µl of a 10mg ml^{-1} DNaseI /RNaseA solution added, and the phage solution shaken at 37°C for 30 minutes. 4ml of a 20% (w/v) polyethylene glycol 6000 (PEG), 2M NaCl solution in phage buffer was added with the phage solution to a 14ml Corex tube (Fisons), mixed well, and kept at 4°C overnight.

The phage /PEG precipitate was pelleted by centrifugation at 10krpm for 20 minutes at 4°C in the SS-34 rotor of a Sorvall centrifuge, and the supernatant removed by aspiration. The phage were resuspended gently in 0.5ml phage buffer. 0.5ml chloroform was added and the phage solution vortexed briefly before being transferred to an Eppendorf tube. The phage were separated from the PEG/chloroform by centrifugation for 30 seconds in a microfuge. The phage-containing aqueous phase was transferred to a fresh Eppendorf tube, 5µl 10% SDS and 5µl 0.5M EDTA pH 8.0 was added and the tube heated to 68°C for 15 minutes. Denatured phage proteins were removed by phenol extraction. A phenol /chloroform and chloroform extraction was followed by two ethanol precipitations to precipitate phage DNA. Phage DNA was finally resuspended in 50µl TE.

7.7.7 Large-scale Phage DNA preparations.

Large-scale preparation of phage DNA was initiated by preparing 200ml or 500ml phage liquid lysates (Section 7.7.4). After addition of chloroform, sodium chloride was added to 40g l^{-1}, and DNaseI /RNaseA (from a 10mg ml^{-1} stock in phage buffer) was added to 1µg ml^{-1}. The lysate was incubated for 1h, then clarified by centrifugation in

the GSA rotor of a Sorvall centifuge, 8 krpm for 10 minutes and titred. Phage were precipitated from solution with PEG. 100g l^{-1} solid PEG was added and left to dissolve for 30 minutes, before transferring the lysates to 4°C for overnight precipitation. The PEG precipitate was pelleted by centrifugation at 10 krpm for 10 minutes in a Sorvall GSA rotor, and was resuspended in 5ml phage buffer per 200ml or 500ml lysate. Resuspension was achieved using a Pasteur pipette, followed by gentle shaking at 4°C for 1-3 hours. PEG was removed by centrifugation at 5 krpm for 5 minutes in a Sorvall GSA rotor, and the phage concentrated on a caesium chloride (CsCl) step gradient.

A saturated solution of 19g CsCl was made up in 10ml phage buffer and 0.5ml was pipetted into a 14ml polycarbonate tube. This was gently overlayed with 0.5ml 2:1 saturated CsCl solution: phage buffer, 1.0ml of 1:1 CsCl solution: phage buffer then 1.5ml of 1:2 CsCl solution: phage buffer. Finally the step gradient was overlayed with the 5ml phage solution. The gradient was centrifuged in a 6 x 14 Ti swing-out rotor of an MSE superspeed 65 ultracentrifuge at 33 krpm (140,000g) for 2 hours at 18°C, and stopped without braking. The single phage band was collected using a 1ml syringe.

Phage were generally purified further on an equilibrium gradient. A 41.5% (w/w) CsCl solution in phage buffer was prepared by addition of 0.71g CsCl g^{-1} of phage buffer and clarified by centrifugation for 1 hour at 12krpm and 4°C in the SS-34 rotor of a Sorvall centrifuge. The phage band from the step gradient was mixed with sufficient CsCl solution to fill a 5ml polycarbonate tube, and centrifuged to equilibrium in a 6 x 5 swing-out rotor in an MSE ultracentrifuge for 22-26 hours at 35 krpm (120,000g) and 4°C.

The phage band was collected and CsCl removed by dialysis against TE for 1 hour at 4°C. The phage proteins were removed by two phenol extractions; the phenol was back-extracted once. Finally phenol was removed by extensive dialysis for at least 24 hours against TE at 4°C. A 200ml lysate generally gave yields of 500µg. (The maximum yield for a 200ml lysate of titre 10^{11} pfu ml^{-1} for a phage comprising 49kbp of DNA is 1.1mg).

7.7.8 Transfection of cells with phage DNA.

Cells were made competent for the uptake of DNA by a modified method of Lederberg and Cohen (1974). A fresh overnight culture of cells (ED8654, C600, 5K or NM514) was diluted 1:100 in LB, and grown with shaking at 37°C until $O.D._{650}$ = 0.6. Cells were left on ice for 15 minutes, pelleted in a bench centrifuge at 2 krpm for 5 minutes, and resuspended in 0.5 the original volume of ice-cold 0.1M $MgCl_2$. The cells were immediately repelleted as previously and resuspended in 0.05 original volume of ice-cold 0.1M $CaCl_2$. The cells were kept on ice for 1-4 hours prior to transfection. DNA was diluted to 0.1ml in a 1 x SSC, 0.1M $CaCl_2$ (ratio 3:4) in a 3ml glass tube and 0.2ml cells were added and incubated on ice for 30 minutes. Cells were subjected to heat shock for four minutes at 42°C and returned to ice for a further 40-60 minutes before plating. The 0.3ml reaction was split into 20µl and 280µl samples before plating out onto 2.5ml BBL top agar supplemented with 10mM $MgSO_4$. Plates were incubated at 37°C overnight.

7.7.9 Packaging of phage (Hohn, 1979; Grosveld *et al.*, 1981).

Phage DNA was packaged by mixing extracts from 2 *E. coli* strains lysogenic for lambda, each of which was deficient for a different phage coat protein. The preparation of these extracts is described below.

7.7.9.1 Preparation of freeze-thaw lysate.

Several confluent plates of BHB2688 grown at 30°C were used to inoculate each of three 500ml aliquots of L broth in

21 flasks. Cultures were grown at 30°C until the OD_{650} reached 0.3. The flasks were then placed in a 45°C water bath for 15 minutes without shaking in order to de-repress the lambda molecules. The cultures were shaken at 37°C until the cells were induced (1-2hours); if chloroform was added to a small sample of cells which were induced, the culture cleared. The cells were cooled on ice then harvested by centrifugation in the GSA rotor of a Sorvall centrifuge at 10 krpm for 10 minutes at 4°C. The supernatants were removed very thoroughly, and the pellets resuspended in 0.5ml cold 10% (w/v) sucrose, 50mM Tris-HCl pH 7.5 per 250ml of culture. The resuspended cells were pooled, and dispensed into two 10ml Oakridge ultracentrifuge tubes. 75µl of a fresh lysosyme solution made up to 2mg ml^{-1} in 0.25M Tris-HCl pH 7.5 was added, and mixed gently but thoroughly. The cell extracts were frozen in liquid nitrogen, then rethawed at 4°C, and placed on ice. 75µl buffer M1 was added per tube, and mixed gently but thoroughly. Cell debris was removed by centrifugation for 35 minutes at 35 krpm (14,000g) and 4°C in a Ti50 rotor of a Sorvall OTD50B ultracentrifuge. The purified extract was aliquoted into pre-cooled Eppendorf tubes, and frozen immediately in liquid nitrogen. Freeze-thaw lysate (FTL) was stored at -70°C.

7.7.9.2 Sonicated extract.

The initial stages of preparation of sonicated extract (SE) were identical to those for the preparation of freeze-thaw lysates, except that plates of BHB2690 grown at 30°C were used to inoculate two 500ml aliquots of L-broth. Following harvesting of the induced cells, the supernatants were removed very thoroughly and the pellets resuspended in 0.5ml buffer A per 250ml culture. The resuspended cells were pooled into one centrifuge bottle. The remaining bottles were rinsed with a total of 2.6ml buffer A, which was added to the pooled cells. The cells were sonicated without foaming ten times for 3 seconds, until the solution was no longer viscous. Cell debris was pelleted by

centrifugation for 10 minutes in a microfuge at 4°C. The supernatants were aliquoted into pre-cooled Eppendorf tubes and frozen immediately in liquid nitrogen. Freeze thaw extract was stored at -70°C.

7.7.9.3 Packaging of phage DNA.

Packaging extracts FTL and SE were thawed on ice for 15-30 minutes. DNA to be packaged (200-500ng) was dried down to 1-2µl in a desiccator if necessary, and 7µl buffer A added. 1µl M1 buffer, 6µl SE and 10µl FTL were then added, mixed gently, and incubated at 25°C for 1-2 hours. (The optimal ratio of SE to FTL varied slightly with the preparation). The packaging reaction was stopped by dilution into 500µl phage buffer. 100µl of 10^{-3} and 10^{-4} dilutions were mixed with 100µl of a fresh overnight culture of cells (supplemented with 10mM $MgSO_4$) and plated onto BBL plates in 2.5ml 10mM $MgSO_4$-supplemented top agar. Packaging efficiencies were in the range of 10^7-1 10^8 pfu μg^{-1}.

7.7.10 Blotting of phage plaques.

Sufficient phage to produce 100-200 plaques per plate were plated onto the appropriate host strain, incubated at 37°C overnight and then cooled at 4°C for 30 minutes. Circular nitrocellulose filters were placed on the phage plates for 2 minutes and alignment marks placed on the filter and the plate. The filters were transferred to four sheets of blotting paper soaked in 0.5M NaOH 1.5M NaCl for 2 minutes to denature phage DNA. They were then rinsed for two minutes in neutralising solution (0.5M Tris-HCl pH 7.4, 3M NaCl) and for a further two minutes in 2 x SSC, air dried and baked at 80°C under vacuum for 2 hours.

7.7.11 The M13, mp18 and pUC13 cloning vectors.

The M13 and pUC13 series of vectors (Messing *et al.*, 1981; Messing, 1983) contain the *lac* Z gene, which encodes a β-galactosidase. When cultured in strain NM522 or JM101 the *lac* Z gene product is repressed by the product of the $lacI^q$ gene carried by the F′ episome. However, growth in

the constitutive inducer isopropyl β-D-thio-galactoside (IPTG) induces β-galactosidase activity; hydolysis of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) gives rise to a blue product and hence blue M13 plaques or pUC13 colonies. Insertion of foreign DNA into the vector using 1 or 2 of the 13 unique restriction endonuclease sites within the *lac* Z gene inactivates the β-galactosidase, and leads to generation of clear M13 plaques or uncoloured colonies bearing pUC plasmids.

7.7.12 Transfection and transformation of NM522 and JM101 cells.

NM522 and JM101 cells were propagated on minimal agar to maintain the F' episome. Competent cells were prepared in a similar manner to that described for phage transfection. A 5ml overnight culture of cells was diluted 1:50 in L-broth and grown to OD_{650} = 0.3. The cells were pelleted by centrifugation at 2krpm in a bench centrifuge for 10 minutes at 4°C, resuspended in 0.5 the original volume of 100mM $CaCl_2$, kept on ice for 20 minutes, repelleted and resuspended in 0.1 the original volume 100mM $CaCl_2$. Cells were kept on ice for 1-4 hours prior to transfection.

DNA (10-50ng) was added to 0.3ml competent cells in a glass tube, incubated on ice for 40-60 minutes and heated to 42°C for 5 minutes. Cells were plated immediately. 2.5ml BBL top agar at 42°C was added to the cells and 30µl 20mg ml^{-1} X-gal, and 20µl 24mg ml^{-1} IPTG (both made up in dimethyl-formamide) were added before plating on minimal or BBL agar.

The high competence strain MC1061 was sometimes used for initial transformations in order to assess the completeness of the digestion and religation of plasmid DNA.

7.7.13 Preparation of M13 phage and infected cells.

Individual M13 plaques were picked into 2ml of OD_{650} = 0.3 NM522 cells in large test-tubes and shaken vigorously for 4-5 hours at 37°C. Cells were pelleted by

centrifugation in a microfuge, and the supernatants containing M13 phage extruded from infected cells were titred and stored at 4°C. 50ml lysates were prepared by growing NM522 cells to OD_{650} = 0.3 in 50ml L broth at 37°C from a 1:100 dilution of an overnight culture and infecting them with a total of 5 10^{10} M13 phage. The infected cells were grown overnight and pelleted by centrifugation at 8krpm for 10 minutes in a Sorvall GSA rotor. The M13 lysates were retained, titred and used to infect 500ml NM522 cells grown to OD_{650} = 1.0. at an moi of 10^{10} pfu ml^{-1}. The cells were grown for a further 2 hours, and then harvested by centrifugation at 8krpm for 10 minutes in a Sorvall GSA rotor.

Preparation of replicative form (RF) DNA was as described in Sections 7.7.14 and 7.7.15 for plasmid DNA.

7.7.14 Small-scale preparation of plasmid/RF DNA (Birnboim and Doly, 1974).

Single colonies were picked into 5ml L-broth supplemented with the appropriate antibiotic and incubated, with shaking, at 37°C for 12-18 hours. Cells from 1.4ml of these cultures were pelleted by centrifugation in a microfuge, and resuspended in 100µl lysis solution (50mM glucose, 25mM Tris-HCl pH 8.0 and 10mM EDTA pH 8.0). Cells were incubated for 5 minutes, 200µl alkaline SDS (0.2M NaOH, 1% (w/v) SDS) added and incubation continued on ice for a further 5 minutes. 150µl pre-cooled 3M KOAc pH 4.8 was added to selectively precipitate bacterial DNA, and incubation was continued for a further 5 minutes. Bacterial DNA and cell debris were pelleted by centrifugation for 2 minutes in a microfuge; a second centrifugation removed any residual debris. Plasmid DNA was precipitated with ethanol at room temperature and pelleted by centriguation in a microfuge.

7.7.15 Large-scale preparation of plasmid DNA.

Two different methods were used to lyse the plasmid -bearing cells, one based on Triton lysis and the other on lysis with alkaline SDS.

5ml overnight cultures of the plasmid-containing cells, grown overnight in the relevant antibiotic from a freshly -plated, single colony, were diluted 1:100 into 500ml antibiotic-supplemented L-broth. Cells were grown to OD_{650} = 1.0, at which point chloramphenicol was added to 50μg ml^{-1}. Cells were incubated at 37°C with shaking overnight, and pelleted by centrifugation at 8,000rpm in the GSA rotor of a Sorvall centrifuge for 10 minutes.

For the method of triton lysis, the cells were resuspended in 6ml sucrose solution (25% (w/v) sucrose, 50mM Tris-HCl pH 8.0 and 40mM EDTA pH 8.0) and 1ml lysozyme, made up at 10mg ml^{-1} in sucrose solution, and 1ml 0.5M EDTA pH 8.0 were then added and the cells incubated on ice for 5 minutes. 13ml of triton mix (0.1% (v/v) triton X-100, 50mM Tris-HCl pH 8.0 and 63mM EDTA pH 8.0) was added and the cells incubated on ice with gentle mixing for a further 10 minutes, during which time the cells lysed. Cellular debris was removed by centrifugation for 40 minutes at 12krpm at 4°C in the HB4 Sorvall rotor and the supernatant extracted once with phenol. The phases were separated by centrifugation for 10 minutes at 8krpm in the HB4 Sorvall rotor. The aqueous phase was isolated and DNA present within it precipitated with ethanol. The DNA precipitate was pelleted by centrifugation at 8krpm and 4°C for 10 minutes, dried and resuspended in 12.5ml TE. 0.1ml of 1mg ml^{-1} RNase A (pre-boiled for 10 minutes to remove any contaminating DNase I) was incubated with the plasmid DNA for 30 minutes at 37°C. 0.5ml of 5mg ml^{-1} ethidium bromide and 0.95g caesium chloride was added per ml of solution. The density of the solution was adjusted to 1.55g ml^{-1} before the plasmid DNA solution was placed in a 14ml heat-seal ultracentrifuge tube. The tubes were sealed and centrifuged at 38krpm (16,000g) in the Ti50 rotor of a Sorvall ultracentrifuge for 48-65 hours.

Following centrifugation, the tubes were viewed under uv light, and the supercoiled plasmid DNA band (lower band) collected with a 2ml syringe. 2 volumes of TE were added to dilute the CsCl, then ethidium bromide was removed by 4 extractions with butan-2-ol. Plasmid DNA was concentrated by ethanol precipitation.

Alkaline-SDS lysis was a large-scale version of the Birnboim-Doly small-scale DNA preparation described above (Maniatis *et al.* 1982). 500ml cells were harvested as described for the triton lysis preparation, and treated with 7ml lysis solution supplemented with 100mg ml^{-1} lysozyme, 14ml alkaline SDS and 6.3ml potassium acetate as described for the small-scale plasmid DNA preparation. Cellular DNA was pelleted by centrifugation at 12krpm in a Sorvall HB4 rotor for 15 minutes at 4°C; a second centrifugation removed any remaining debris. Plasmid DNA was precipitated by addition of 0.5 volume isopropanol, and pelleted by centrifugation at 8krpm in a Sorvall HB4 rotor for 10 minutes. The pellet was resuspended in TE, phenol extracted, and re-precipitated with ethanol. Preparation of ethidium-bromide caesium-chloride gradients was as described for plasmid DNA preparation by triton lysis.

7.8 Sequencing techniques.

7.8.1 Preparation of M13 templates.

Fresh, well isolated M13 plaques grown overnight on BBL plates were picked, using a tooth-pick, into 2 ml of a 1:100 dilution of a fresh 5ml overnight culture of NM522 or JM101 cells. The cells were incubated for 4-6 h with vigorous shaking at 37°C. Single stranded extracellular phage were separated by centrifugation in a microfuge for 5 minutes. The cell pellets were discarded or used to make phage RF DNA preparations and 1.5 ml of the supernatants containing the single stranded phage were added to 200 µl of 20% (w/v) PEG in 2.5M NaCl. Following a 30 minute incubation the phage were pelleted by centrifugation for 5 minutes in a microfuge. The supernantants were removed by aspiration;

this step was repeated to ensure removal of all unbound PEG. 100 µl TE and 50 µl phenol was added to the phage pellets, which were resupended and the PEG removed by a 10 second vortex, incubation for 10 minutes and a further 10 second vortex. Following a 1 minute centrifugation in a microfuge, 85 µl of the aqueous phase was removed and M13 DNA precipitated by addition of 10 µl 3M NaOAc and 250 µl ethanol. The DNA was immediately frozen in an ethanol/dry ice bath and pelleted by centrifugation for 10 minutes in a microfuge. The pellets were washed with ethanol, dried, resuspended in 50 µl TE and stored at -20°C.

7.8.2 Sequencing reactions

Sequencing by the dideoxy chain-termination method of Sanger *et al.* (1977 and 1980) was performed using the BRL T7 sequencing kit. Reactions were performed as instructed by the manufacturer.

7.8.3 Preparation and running of sequencing gels.

Sequencing gels were poured between 30 x 40 cm plates which formed a part of the BRL sequencing apparatus. The plates were prepared by sequential washing in distilled water and ethanol. 8% (w/v) acrylamide gels were prepared using 7.5 ml 10x sequencing TBE, 37.5 g urea, 15 ml of a 40% (w/v) stock of 19:1 acrylamide: bisacrylamide and 22.5 ml distilled water; incubation at 37°C for 1 hour was required to dissolve the urea. 750 µl freshly prepared 10% (w/v) ammonium persulphate and 12 µl TEMED were added immediately prior to pouring the gel. The gel was poured using an inverted sharks tooth comb to create a flat surface to the gel and was left to set for at least 1 hour.

Freshly denatured DNA samples in formamide dye were added to the wells created by the sharks tooth comb. Samples were electrophoresed at 50W (1200V) for multiples of 3 h, the time it took the bromophenol blue to travel through the gel.

7.8.4 Analysis of sequence data.

Sequence information was compared with that in the EMBL data bank using the Wisconsin package (Devereux *et al.*, 1984) available through "Agrenet", the Agriculture and Food Research (computing) Network.

7.9 Virological methods.

7.9.1 Cultivation of virus.

The WC11 isolate of AHV-1 was propagated in monolayer cultures of bovine kidney cells grown in roller vessels in 199 medium (Gibco) supplemented with 5% (v/v) foetal bovine serum (FBS). Supernatant fluids which contained approximately 5 10^6 $TCID_{50}$ per ml were harvested when about half of the cells showed cytopathic effects (cpe). The supernatant was clarified by centrifugation at 9 krpm (9000g) for 20 minutes at 4°C and the virus pelleted by centrifugation at 19 krpm (40,000g) in a 100ml fixed-angle rotor at 5°C. Pellets were resuspended in 8ml of TNE buffer pH 7.5, loaded on a preformed 7.5 ml 15-40% (w/w) Metrizamide (Nyegaard, UK Ltd.) gradient in TNE pH 7.5 , and centrifuged at 30 krpm (114,000g) for 60 minutes at 4°C in a SW40 rotor. The virus band was collected, diluted to 12 ml in TNE and repelleted at 30 krpm for 40 minutes at 4°C. Viral pellets were resuspended in 100µl TNE and the purity of the preparation assessed by electron microscopy (25,000 fold magnification) after negative staining with 1% phosphotungstate.

7.9.2 Preparation of viral DNA.

Viral pellets were diluted to 850µl in TNE pH 8.0, SDS was added to 1% (w/v), and proteinase K added to 200µg ml^{-1} and incubation performed for 2-4 hours at 37°C. Protein was removed by phenol extraction, phenol was extracted with chloroform and the viral DNA was precipitated with ethanol using mussel glycogen carrier.

7.9.3 Preparation of viral DNA from infected cells.

DNA from the cell-associated C500 isolate was obtained as follows. Virus-susceptible cells (bovine thyroid) were co-cultivated with cells from the lymph nodes of a C500-infected rabbit. The cultures were washed after 24 hours and the rabbit cells discarded. When cytopathic effects affected approximately 20% of the monolayer the cells were harvested, washed in PBS and lysed by resuspension in 0.5% (v/v) NP40 in RSB (RSB= 10mM NaCl, 1mM $MgCl_2$, 10mM Tris-HCl pH 7.5) using gentle homogenisation. Chromatin and cellular debris were removed by centrifugation for 10 minutes at 800 rpm in a bench centrifuge at 4°C. The supernatant was layered over 25% (w/v) sucrose in RSB and viral nucleocapsids pelleted by centrifugation at 30 krpm (114,000g) in a Beckman SW40 rotor at 4°C. Viral DNA was then prepared from the pellet using the selective extraction procedure of Hirt (1967). The supernatant fraction from this extraction was digested with proteinase K and phenol extracted, and the DNA precipitated with ethanol.

7.9.4 Estimation of viral infectivity.

Tissue pieces were finely chopped and suspended in 10 ml Iscoves medium (Gibco) supplemented with 10% FBS, 50 units ml^{-1} streptomycin, 50 units ml^{-1} penicillin and 0.06% (v/v) β-mercaptoethanol prior to dissaggregation in a Stomacher 400 (Seward Laboratory). The cell suspension was carefully removed from the tissue debris, sedimented at 2krpm in a bench centrifuge, resuspended in Iscoves medium containing 10% dimethylsulphoxide (DMSO) and 20% FBS and stored at -80°C.

100µl of sequential 10 fold dilutions of the cells were added to each of 6 preformed bovine thyroid monolayer cultures in microtitre plates. The medium was changed on the 7th day and the plates examined for evidence of cpe. On day 14 the medium was discarded and the monolayers were washed 3 times in PBS and fixed in cold acetone. Monolayers were then treated with a 1:350 dilution of a pool of 5 monoclonal

antibodies to WC11 followed by rabbit anti-mouse immunoglobulin. Monolayers were further examined under uv.

The $TCID_{50}$ for each specimen was calculated by the Karber method and the result expressed as $TCID_{50}$ per 10^6 cells. The limit of sensitivity was approximately 10 $TCID_{50}$ per 10^6 cells.

7.10 Electron microscopy.

DNA samples were spread in the presence of cytochrome C and pAT153 and M13 size standards in the solution described. Some samples were partially denatured for 75 seconds, 5 minutes or 20 minutes and neutralised in the sodium carbonate solution described prior to spreading. Samples were viewed using a Siemens Elmiscop 1A microscope, photographed, and the molecule lengths estimated from the photographs using a Ferranti Cetec tablet digitiser linked to an Olivetti P6040 programmable calculator.

References

Ablashi, D.V., Schirm, S., Fleckenstein, B., Faggioni, A., Dahlberg, J., Rabin, H., Loeb, W., Armstrong, G., Whang Peng, J., Aulahk, G. and Torrisi, M.R. (1985). *Herpesvirus saimiri*-induced lymphoblastoid rabbit cell line: growth characteristics, virus persistence, and oncogenic properties. *Journal of Virology* **55**, 623-633.

Adams, A. and Lindahl, T. (1975). Epstein-Barr virus genomes with properties of circular DNA molecules in carrier cells. *Proceedings of the National Academy of Sciences* **72**, 1477-1481.

Ades E.W. and Lopez, C. (Eds.)(1989). Natural killer cells and host defense. *5th International Natural Killer Cell Workshop, 1988*. Basel and worldwide: Karger.

Appleyard, R.K. (1954). Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. *Genetics* **39**, 440-452.

Baer, R., Bankier, A.T., Biggin, M.D., Deininger, P.L., Farrell, P.J., Gibson, T.J., Hatfull, G., Hudson, G.S., Satchwell, S.C., Seguin, C., Tuffnell, P.S. and Barrell, B.G. (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* **310**, 207-211.

Bankier, A.T., Dietrich, W., Baer, R. Barrel, B.G., Colbere-Garapin, F., Fleckenstein, B. and Bodemer, W. (1985). Terminal repetitive sequences in *Herpesvirus saimiri* virion DNA. *Journal of Virology* **55**, 133-139.

Barnard, B.J.H. (1985). Transmitters and potential transmitters of malignant catarrhal fever. *Proceedings of the XIIIth World Congress on diseases in cattle*. Durban, September 17th-21st, 1984.

Bartha, A., Fadol, A.M., Liebermann, H., Ludwig, H., Mohanty, S.B., Osorio, F.A., Reed, D.E., Storz, J., Straub, O.C., van der Maaten, M.J. and Wellemans, G. (1987). Problems concerning the taxonomy of the "Movar-Type" bovine herpesviruses. *Intervirology* **28**, 1-7.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513-1523.

Bodemer, W., Knust, E., Angermuller, S. and Fleckenstein, B. (1984). Immediate-early transcription of *Herpesvirus saimiri*. *Journal of Virology* **51**, 452-457.

Bornkamm, G.W., Delius, H., Fleckenstein, B., Werner, F-J. and Mulder, C. (1976). Structure of *Herpesvirus saimiri* genomes: arrangement of heavy and light sequences in the M genome. *Journal of Virology* **19**, 154-161.

Bouliere, F. (Ed., 1983). "Ecosystems of the World 13: Tropical Savannas". Elsevier Scientific Publishing Co. p422.

Brahic, M. and Haase, A.T. (1978). Detection of viral sequences of low reiteration frequency by *in situ* hybridization. *Proceedings of the National Academy of Sciences* **75**,6125-6129.

Bridgen, A., Herring, A.J., Inglis, N.F. and Reid, H.W. (1989). Preliminary characterization of the *Alcelaphine herpesvirus* 1 genome. *Journal of General Virology* **70**, 1141-1150.

Brown, F. (1986). The classification and nomenclature of viruses; summary of results of meetings of the International Committee on Taxonomy of Viruses in Sendai, September 1984. *Intervirology* **25**, 141-143.

Buckmaster, A.E., Scott, S.D., Sanderson, M.J., Boursnell, M.E.G., Ross, N.L.J. and Binns, M.M. (1988). Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implications for herpesvirus classification. *Journal of General Virology* **69**, 2033-2042.

Buxton, D. and Reid, H.W. (1980). Transmission of malignant catarrhal fever to rabbits. *Veterinary Record* **106**, 243-245.

Buxton, D., Reid, H.W., Finlayson, J. and Pow, I. (1984). Pathogenesis of "sheep-associated" malignant catarrhal fever in rabbits. *Research in Veterinary Science* **36**, 205-211

Cameron, K.R., Stamminger, T., Craxton, M., Bodemer, W., Honess, R.W. and Fleckenstein, B. (1987). The 160,000-Mr virion protein encoded at the right end of the *Herpesvirus saimiri* genome is homologous to the 140,000-Mr membrane antigen encoded at the left end of the Epstein-Barr virus genome. *Journal of Virology* **61**, 2063-2070.

Carle, G.F. and Olson, M.V. (1984). Separation of chromosomal DNA molecules from yeast by orthogonal-field-alteration gel electrophoresis. *Nucleic Acids Research* **12**, 5647-5664.

Casadaban, M.J. and Cohen, S.N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *Journal of Molecular Biology* **138**, 179-207.

Castro, A.E., Daley, G.G., Zimmer, M.A., Whitenack, D.L. and Jensen, J. (1982). Malignant catarrhal fever in an Indian gaur and greater kudu: experimental transmission, isolation, and identification of a herpesvirus. *American Journal of Veterinary Research* **43**, 5-11.

Clark, K.A. and Adams, L.G. (1976). Viral particles associated with malignant catarrhal fever in deer. *American Journal of Veterinary Research* **37**, 837-840.

Clarke, J.K., McFerran, J.B. and Nelson, R.T. (1973). The isolation of a strain of bovine syncitial vius in Northern Ireland. *Research in Veterinary Science* **14**, 117-119.

Cohrs, R. and Rouhandeh, H. (1987). Characterization of the genome of *Herpesvirus sylvilagus*. *Intervirology* **28**, 181-184.

Coulter, G.R. and Storz, J. (1979). Identification of a cell -associated morbillovirus from cattle affected with malignant catarrhal fever; antigenic differentiation and cytological characterization. *American Journal of Veterinary Research* **40**, 1671-1677.

Cox, K.H., Deleon, D.V., Angerer, L.M. and Angerer, R.C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Development Biology* **101**, 485-502.

Daniels, P.W., Sudarisman and Ronohardjo, P. (Eds., 1988). "Malignant Catarrhal Fever in Asian livestock". Canberra: Australian Centre for International Agricultural Research.

Daubney, R. and Hudson, J.R. (1936). Transmission experiments with bovine malignant catarrh. *Journal of Comparative Pathology* **49**, 63-89.

Davison, A.J. and Scott, J.E. (1986). The complete DNA sequence of Varicella -Zoster Virus. *Journal of General Virology* **67**, 1759-1816.

Denholm, L.J. and Westbury, H.A. (1982). Malignant catarrhal fever in farmed Rusa deer (*Cervus timorensis*). 1. Clinico -pathological observations. *Australian Veterinary Journal* **58**, 81-87.

Desrosiers, R.C. (1981). *Herpesvirus saimiri* DNA in tumor cells-deleted sequences and sequence rearrangements. *Journal of Virology* **39**, 497-509.

Desrosiers, R.C., Burghoff, R.L., Bakker, A. and Kamine, J. (1984). Construction of replication-competent *Herpesvirus saimiri* deletion mutants. *Journal of Virology* **49**, 343-348.

Desrosiers, R.C., Bakker, A., Kamine, J., Falk, L.A., Hunt, R.D. and King, N.W. (1985). A region of the *Herpesvirus saimiri* genome required for oncogenicity. *Science* **228**, 184-187.

Desrosiers, R.C., Silva, D.P., Waldron, L.M. and Letvin, N.L. (1986). Nononcogenic deletion mutants of *Herpesvirus saimiri* are defective for *in vitro* immortalization. *Journal of Virology* **57**, 701-705.

Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research*. **12**, 387-395.

Ebeling, A., Keil, G.M., Knust, E. and Koszinowski. U.H. (1983). Molecular cloning and physical mapping of murine cytomegalovirus DNA. *Journal of Virology* **47**, 421-433.

Edington, N. and Plowright, W. (1980). The protection of rabbits against the herpesvirus of malignant catarrhal fever by inactivated vacccines. *Research in Veterinary Science* **28**, 384-386.

Edington, N. and Patel, J.R. (1981). The location of primary replication of the herpesvirus of bovine malignant fever in rabbits. *Veterinary Microbiology* **6**, 107-112.

Edington, N., Patel, J., Russell, P.H. and Plowright, W. (1979). The nature of the acute lymphoid proliferation in rabbits infected with the herpes virus of bovine malignant catarrhal fever. *European Journal of Cancer* **15**, 1515-1522.

Ehlers, B., Buhk, H-J. and Ludwig, H. (1985). Analysis of bovine cytomegalovirus genome structure: cloning and mapping of the monomeric polyrepetitive DNA unit, and comparison of European and American strains. *Journal of General Virology* **66**, 55-68.

Engels, M., Loepfe, E., Wild, P., Schraner, E. and Wyler, R. (1987). The genome of caprine herpesvirus 1: genome structure and relatedness to bovine herpesvirus 1. *Jounal of General Virology* **68**, 2019-2023.

Falk, L.A. (1980a). Simian herpesviruses and their oncogenic properties. In "Oncogenic Herpesviruses" pp.145-173. Edited by F. Rapp. Chicago: CRC Press.

Falk, L.A. (1980b). Biology of *Herpesvirus saimiri* and *Herpesvirus ateles*. In "Viral Oncology", pp.813-832. Edited by G. Klein. New York: Raven Press.

Falk, L., Wright, J., Wolfe, L. and Deinhardt, F. (1974). *Herpesvirus ateles*: transformation *in vitro* of marmoset splenic lymphocytes. *International Journal of Cancer* **14**, 244-251.

Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6-13.

Feinberg, A.P. and Vogelstein, B. (1984). Addendum. *Analytical Biochemistry* **137**, 266-267.

Fleckenstein, B. and Desrosiers, R.C. (1982). *Herpesvirus saimiri* and *Herpesvirus ateles*. In "The Herpesviruses" Vol. 1 pp253-332. Edited by B. Roizman. New York and London: Plenum Press.

Fleckenstein, B. and Mulder, C. (1980). Molecular biological aspects of *Herpesvirus saimiri* and *Herpesvirus ateles*. In "Viral Oncology" pp.799-812. Edited by G. Klein. New York: Raven Press.

Fleckenstein, B., Bornkamm, G.W. and Ludwig, H. (1975). Repetitive sequences in complete and defective genomes of *Herpesvirus saimiri*. *Journal of Virology* **15**, 398-406.

Fleckenstein, B., Muller, I. and Werner, J. (1977). The presence of *Herpesvirus saimiri* genomes in virus-transormed cells. *International Journal of Cancer* *19*, 546-554.

Fleckenstein, B. Bornkamm, G.W., Mulder, C., Werner, F-J. Daniel, M.D., Falk, L.A. and Delius, H. (1978). *Herpesvirus ateles* DNA and its homology with *Herpesvirus saimiri* nucleic acid. *Journal of Virology* **25**, 361-373.

Frischauf, A-M., Lehrach, H., Poustka, A. and Murray, N. (1983). Lambda replacement vectors carrying polylinker sequences. *Journal of Molecular Biology* **170**, 827-842.

Fuchs, P.G., Ruger, R., Pfister, H. and Fleckenstein, B. (1985). Genome organization of *Herpesvirus Aotus* type 2. *Journal of Virology* **53**, 13-18.

Gardella, T., Medveczky, P., Sairenji, T. and Mulder, C. (1984). Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. *Journal of Virology* **50**, 248-254.

Goetze, R (1930). Untersuchungen uber das bosartige Katarrhalfieber des Rindes. *Deutsch. Tier. Woch.* **38**, 487-491 (descibed in the Veterinary Bulletin, 1931, **1**, 89-90).

Gompels, U.A., Craxton, M.A. and Honess, R.W. (1988). Conservation of gene organization in the lymphotropic herpesviruses *Herpesvirus saimiri* and Epstein-Barr Virus. *Journal of Virology* **62**, 757-767.

Gompels, U.A., Craxton, M.A. and Honess, R.W. (1989). Conservation of glycoprotein H (gH) in herpesviruses: nucleotide sequence of the gH gene from *Herpesvirus saimiri*. *Journal of General Virology* **69**, 2819-2829.

Goodheart, C.R. and Plummer, G. (1975). The densities of herpesviral DNAs. *Progress in Medical Virology* **19**, 324-352.

Gough, J.A. and Murray, N.E. (1983). Sequence diversity among related genes for recognition of specific targets in DNA molecules. *Journal of Molecular Biology* **166**, 1-20.

Grosveld, F.G., Dahl, H-Hm, de Boer, E. and Flavell, R.A. (1981). Isolation of β-globin-related genes from a human cosmid library. *Gene* **13**, 227-237.

Haase, A., Brahic, M., Stowring, L. and Blum, H. (1984). Detection of viral nucleic acids by *in situ* hybridization. *Methods in Virology* **7**, 189-226.

Hambdy, F.M., Dardiri, A.H., Mebus, C., Pierson, R.E. and Johnson, D. (1978). Etiology of malignant catarrhal fever outbreak in Minnesota. *Proceedings of the 82nd Annual Meeting of the United States Animal Health Association*, Buffalo, New York, pp248-267.

Hamblin, C. and Hedger, R.S. (1984). Neutralising antibodies to wildebeest derived malignant catarrhal fever virus in African wildlife. *Comparative Immunology and Microbiology of Infectious Diseases* **7**, 195-199.

Hatkin, J. (1980). Endemic malignant catarrhal fever at the San Diego Wild Animal Park. *Journal of Wildlife Diseases* **16**, 439-443.

Herring, A.J., Inglis, N.F., Ojeh, C.K., Snodgrass, D.R. and Menzies, J.D. (1982). Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *Journal of Clinical Microbiology* **16**, 473-477.

Herring, A.J., Reid, H., Inglis, N. and Pow, I. (1989). Immunoblotting analysis of the reaction of wildebeest, sheep and cattle sera with the structural antigens of *Alcelaphine herpesvirus* 1 (Malignant Catarrhal Fever virus). *Veterinary Microbiology* **19**, 205-215.

Heuschele, W.P. (1982). Malignant catarrhal fever in wild ruminants-a review and current status report. *Proceedings of the 86th Annual Meeting of the U.S. Animal Health Association* pp552-570.

Heuschele, W.P., Fletcher, H.R., Oosterhuis, J., Jannsen, D. and Robinson, P.T. (1984). Epidemiologic aspects of malignant catarrhal fever in the USA. *Proceedings of the 88th Annual Meeting of the U.S. Animal Health Association* pp640-651.

Heuschele, W.P., Seal, B.S. and Klieforth, R.B. (1988). Malignant catarrhal fever in wild ruminants. Internationalen Symposiums uber die Erkrankungen der Zoo- und Wildtiere. Sofia.

Hinze, H.C. (1971). New member of the herpesvirus group isolated from wild cottontail rabbits. *Infection and Immunity* **3**, 350-354.

Hirai, K., Ikuta, K., Maotani, K. and Kato, S. (1984). Evaluation of DNA homology of Marek's Disease virus, herpesvirus of turkeys and Epstein-Barr virus under varied stringent hybridization conditions. *Journal of Biochemistry* **95**, 1215-1218.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *Journal of Molecular Biology* **26**, 365-369.

Hohn, B. (1979). *In vitro* packaging of lambda and cosmid DNA. *Methods in Enzymology* **68**, 299-309.

Honess, R.W. (1984). *Herpes simplex* and "The Herpes complex': diverse observations and a unifying hypothesis. *Journal of General Virology* **65**, 2077-2107.

Honess, R.W. and Watson, D.H. (1977). Unity and diversity in the Herpesviruses. *Journal of General Virology* **37**, 15-37.

Hubacek, J. and Glover, S.W. (1970). Complementation analysis of temperature-sensitive host specificity mutations in *Escherichia coli*. *Journal of Molecular Biology* **50**, 111-127.

Huck, R.A., Shand, A., Allsop, P.J. and Paterson, A.B. (1961). Malignant catarrhal fever of deer. *Veterinary Record* **19**, 457-465.

Hunt, R.D. and Billups, L.H. (1979). Wildebeest-associated Malignant Catarrhal Fever in Africa: A neoplastic disease of cattle caused by an oncogenic herpesvirus? *Comparative Immunology and Microbiology of Infectious Diseases* **2**, 275-283.

Hyman, R.W., Brunovskis, I. and Summers, W.C. (1973). DNA base sequence homology between coliphages T7 and λII and between T3 and λII as determined by heteroduplex mapping in the electron microscope. *Journal of Molecular Biology* **77**, 189-196.

Jacoby, R.O., Reid, H.W., Buxton, D. and Pow, I. (1988a). Transmission of wildebeest-associated and sheep-associated Malignant Catarrhal Fever to hamsters, rats and guinea-pigs. *Journal of Comparative Pathology* **98**, 91-98.

Jacoby, R.O., Buxton, D. and Reid, H.W. (1988b). The pathology of wildebeest -associated malignant catarrhal fever in hamsters, rats and guinea-pigs. *Journal of Comparative Pathology* **98**, 99-109.

Johnson, D.R. and Jondal, M. (1981). Herpesvirus-transformed cytotoxic T-cell lines. *Nature* **291**, 81-83.

Johnson, D.R., Ohno, S., Kashka-Dierich, C., Fleckenstein, B. and Klein, G. (1981). Relationship between *Herpesvirus ateles*-associated nuclear antigen (HATNA) and the number of virus genome equivalents in HVA-carrying lymphoid lines. *Journal of General Virology* **52**, 221-226.

Kaiser, K. and Murray, N.E. (1985). The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries. In "DNA Cloning" volume I pp1-47. Edited by D.M. Glover. Oxford: IRL Press Limited.

Kaschka-Dierich, C., Adams, A., Lindahl, T., Bornkamm, G.W., Bjursell, G., Klein, G., Giovanella, B.C. and Singh, S. (1976). Intracellular forms of Epstein-Barr virus DNA in human tumour cells *in vivo*. *Nature* **260**, 302-306.

Kaschka-Dierich, C., Werner, F-J., Bauer, I. and Fleckenstein, B. (1982). Structure of nonintegrated, circular *Herpesvirus saimiri* and *Herpesvirus ateles* genomes in tumor cell lines and *in vitro-transformed* cells. *Journal of Virology* **44**, 295-310.

Kiyotaki, M., Desrosiers, R.C. and Letvin, N.L. (1986). *Herpesvirus saimiri* strain 11 immortalizes a restricted marmoset T8 lymphocyte subpopulation *in vitro*. *Journal of Experimental Medicine* **164**, 926-931.

Kiyotaki, M., Solomon, K.R. and Letvin, N.L. (1988). *Herpesvirus ateles* immortalizes in vitro a $CD3^+$ $CD4^+$ $CD8^+$ marmoset lymphocyte with NK function. *Journal of Immunology* **140**, 730-736.

Knust, E. Dietrich, W., Fleckenstein, B. and Bodemer, W. (1983a). Virus-specific transcription in a *Herpesvirus saimiri*-transformed lymphoid tumor cell line. *Journal of Virology* **48**, 377-383

Knust, E., Schirm, S., Dietrich, W., Bodemer, W., Kolb, E. and Fleckenstein, B. (1983b). Cloning of *Herpesvirus saimiri* DNA fragments representing the entire L-region of the genome. *Gene* **25**, 281-289.

Koch, H-G., Delius, H., Matz, B., Flugel, R.M., Clarke, J. and Darai, G. (1985). Molecular cloning and physical mapping of the Tupaia Herpesvirus genome. *Journal of Virology* **55**, 86-95.

Kramp,W.J., Medveczky, P., Mulder, C., Hinze, H.C. and Sullivan, J.L. (1985). *Herpesvirus sylvilagus* infects both B and T lymphocytes *in vivo*. *Journal of Virology* **56**, 60-65.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lederberg, E.M. and Cohen, S.N. (1974). Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. *Journal of Bacteriology* **119**, 1072-1074.

Lee. G.M., Thornthwaite, J.T. and Rasch, E.M. (1984). Picogram per cell determination of DNA by flow cytofluorometry. *Analytical Biochemistry* **137**, 221-226.

Liggitt, H.D. and deMartini, J.C. (1980a). The pathomorphology of malignant catarrhal fever. I. Generalized lymphoid vasculitis. *Veterinary Pathology* **17**, 58-72.

Liggitt, H.D. and deMartini, J.C. (1980b). The pathomorphology of malignant catarrhal fever. II. Multisystemic epithelial lesions. *Veterinary Pahology* **17**, 73-83.

Liggitt, H.D., McChesney, A.E. and DeMartini, J.C. (1980). Experimental transmission of bovine malignant catarrhal fever to a bison (*Bison bison*). *Journal of Wildlife Diseases* **16**, 299-304.

Loening, U. E. (1969). The determination of the molecular weight of ribonucleic acid by polyacrylamide-gel electrophoresis. *Biochemical Journal* **113**, 131-138.

Ludwig, H. (1983). Bovine herpesviruses. In "The Herpesviruses" volume 2 p.135-214. Edited B. Roizman. New York and London: Plenum Press.

Lupke, H.F. (1906). Ueber periarteritis nodosa bei Axishirschen. *Verhandlungen Deutschen Pathologsch Gessellschaft* **10**, 149-159.

McClenaghan, M., Herring, A.J., and Aitken, I.D. (1984). Comparison of *Chlamydia psittaci* isolates by DNA restriction endonuclease analysis. *Infection and Immunity* **45**, 384-389.

McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E. and Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of Herpes Simplex Virus Type 1. *Journal of General Virology* **69**, 1531-1574.

Mackay, J.M.C. (1969). Tissue culture studies of sheep pulmonary adenomatosis (Jaagsiekte). I. Direct cultures of affected lungs. *Journal of Comparative Pathology* **79**, 141-146.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). "Molecular Cloning: A laboratory Manual" New York: Cold Spring Harbor Laboratory.

Medveczky, P., Szomolanyi, E., Desrosiers, R.C. and Mulder, C. (1984). Classification of *Herpesvirus saimiri* into three groups based on extreme variation in a DNA region required for oncogenicity. *Journal of Virology* **52**, 938-944.

Medveczky, M.M., Geck, P., Clarke, C., Byrnes, J., Sullivan, J.L. and Medveczky, P.G. (1989a). Arrangement of repetitive sequences in the genome of *Herpesvirus sylvilagus*. *Journal of Virology* **63**, 1010-1014.

Medveczky, M.M., Szomolanyi, E., Hesselton, R., Degrand, D., Geck, P. and Medveczky, P.G. (1989b). *Herpesvirus saimiri* strains from three DNA subgroups have different oncogenic potentials in New Zealand white rabbits. *Journal of Virology* **63**, 3601-3611.

Meinkoth, J. and Wahl, G. (1984). Hybridization of nucleic acids immobilized on solid suports. *Analytical Biochemistry* **138**, 267-284.

Messing, J. (1983). New M13 vectors for cloning. *Methods in Enzymology* 101, 20-78. Academic Press: New York.

Messing, J., Crea, R. and Seeburg, P.H. (1981). A system for shotgun DNA sequencing. *Nucleic Acids Research* **9**, 309-321.

Mettam, R.W.M. (1923). Snotsiekte in cattle. *9th and 10th reports of the Director of Veterinary Education and Research, Union of South Africa* pp395-432.

Murray, N.E. (1983a). Phage Lambda and Molecular Cloning. In "Lambda II" pp395-432. New York: Cold Spring Harbor Laboratory.

Murray, N.E. (1983b). Appendix III. In "Lambda II" pp677-684. New York: Cold Spring Harbor Laboratory.

Murray, N.E., Brammar, W.J. and Murray, K. (1977). Lambdoid phages that simplify the recovery of in vitro recombinants. *Molecular and General Genetics* **150**, 53-61.

Murthy, S.C.S, Trimble, J.J. and Desrosiers, R.C. (1989). Deletion mutants of *Herpesvirus saimiri* define an open reading frame necessary for transformation. *Journal of Virology* **63**, 3307-3314.

Mushi, E.Z., Karstad, L. and Jessett, D.M. (1980). Isolation of bovine malignant catarrhal fever virus from ocular and nasal secretions of wildebeest calves. *Research in Veterinary Science* 29, 168-171.

Mushi, E.Z., Rossiter, P.B., Jessett, D. and Karstad, L. (1981). Isolation and characterization of a herpesvirus from topi (*Damaliscus korrigum*, Ogilby). *Journal of Comparative Pathology* 91, 63-68.

Neubauer, R.H., Dunn, F.E. and Rabin, H. (1981). Infection of multiple T-cell subsets and changes in lymphocyte functions associated with *Herpesvirus saimiri* infection of owl monkeys. *Infection and Immunity* 32, 698-706.

Norrander, J., Kempe, T. and Messing, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* 26, 101-106.

Oliver, R.E., Beatson, N.S., Cathcart, A. and Poole, W.S. (1983). Experimental transmission of Malignant Catarhal Fever to red deer (*Cervus elaphus*). *New Zealand Veterinary Journal* 31, 209-212.

Olson, M.V., Loughney, K. and Hall, B.D. (1979). Identification of the yeast DNA sequences that correspond to specific tyrosine -inserting nonsense suppressor loci. *Journal of Molecular Biology* 132, 387-410.

Ortaldo, J.R., Neubauer, R.H., Blanca, I. and Rabin, H. (1985). Cytotoxic activity of normal and *Herpesvirus saimiri*-transformed nonhuman primate cells. *Cellular Immunology* 95, 349-357.

Osorio, F.A. and Reed, D.A. (1983). Experimental inoculation of cattle with bovine herpesvirus-4: Evidence for a lymphoid -associated persistent infection. *American Journal of Veterinary Research* 44, 975-980.

Osorio, F.A., Reed, D.E., Van der Maaten, M.J. and Metz, C.A. (1985). Comparison of the herpesviruses of cattle by DNA restriction endonuclease analysis and serologic analysis. *American Journal of Veterinary Research* **46**, 2104-2109.

Patel, J.R. and Edington, N. (1980). The detection of the herpesvirus of bovine malignant catarrhal fever in rabbit lymphocytes *in vivo* and *in vitro*. *Journal of General Virology* **48**, 437-444.

Patel J.R. and Edington, N. (1981). The detection and behaviour of the herpesvirus of malignant catarrhal fever in bovine lymphocytes. *Archives of Virology* **68**, 321-326.

Patel, J.R. and Edington, N. (1982). The effect of antibody and complement on the expression of the herpesvirus of bovine malignant fever in cultured rabbit lymphocytes. *Veterinary Microbiology* **7**, 325-333.

Piercey, S.E. (1955). Studies in bovine malignant catarrh. VI. Adaptation to rabbits. *British Veterinary Journal* **111**, 484-491.

Pierson, R.E., Thake, D., McChesney, A.E. and Storz, J. (1973). An epizootic of malignant catarrhal fever in feedlot cattle. *Journal of the American Veterinary Medical Association* **163**, 349-350.

Pierson, R.E., Hambdy, F.M., Dardiri, A.H., Ferris, D.H. and Schloer, G.M. (1979). Comparison of African and American forms of malignant catarrhal fever: transmission and clinical signs. *American Journal of Veterinary Research* **40**, 1091-1095.

Plowright, W. (1965). Malignant catarrhal fever in East Africa. I-Behaviour of the virus in free -living populations of blue wildebeest (*Gorgon taurinus taurinus*, Burchell). *Research in Veterinary Science* **6**, 56-68

Plowright, W. (1967). Malignant Catarrhal Fever in East Africa III - neutralizing antibody in free-living wildebeest. *Research in Veterinary Science* **8**, 129-136.

Plowright, W. (1968). Malignant Catarrhal Fever. *Journal of the American Veterinary Medical Association* **152**, 795-804.

Plowright, W. (1982). Malignant Catarrhal Fever Virus: a lymphotropic herpesvirus of ruminants. In "Latent Herpesvirus Infections in Veternary Medicine" pp279-305. Edited by G. Wittman, R. Gaskell and H. Ryiha. Boston and Hague: Martinus Nijhoff.

Plowright, W., Ferris, R.D. and Scott, G.R. (1960). Blue wildebeest and the aetiological agent of Bovine Malignant Catarrhal Fever. *Nature* **188**, 1167-1169.

Plowright, W., Macadam, R.F. and Armstrong, J.A. (1963). Growth and characterization of the virus of bovine Malignant Catarrhal Fever in East Africa. *Journal of General Microbiology* 39, 253-266.

Plowright, W., Kalunda, M., Jessett, D.M., Herniman, K.A.J. (1972). Congenital infection of cattle with the herpesvirus causing malignant catahhal fever. *Research in Veterinary Science* **13**, 37-45.

Plowright, W., Herniman, K.A.J., Jessett, D.M., Kalunda, M. and Rampton, C.S. (1975). Immunisation of cattle against the herpesvirus of malignant catarrhal fever; failure of inactivated vaccines with adjuvant. *Research in Veterinary Science* **19**, 159-166.

Pooijen-Knegt, van A.C., Raap, A.K., Burg, van der M.J.M., Vrolijk, J. and Ploeg, van der M. (1982). Spreading and staining of human metaphase chromosomes on aminoalkylsilane-treated glass slides. *Histochemical Journal* **14**, 333-344.

Rabin, H., Hopkins, R.F., Desrosiers, R.C., Ortaldo, J.R., Djeu, J.Y. and Neubauer, R.H. (1984). Transformation of owl monkey T cells *in vitro* with *Herpesvirus saimiri*. *Proceedings of the National Academy of Sciences* **81**, 4563-4567.

Rackwitz, H-R., Zehetner, G., Frischauf, A-M. and Lehrach, H. (1984). Rapid restriction mapping of DNA cloned in lambda phage vectors. *Gene* **30**, 195-200.

Reid, H.W. and Rowe, L. (1973). The attenuation of a Herpes virus (Malignant Catarrhal Fever virus) isolated from Hartebeest (*Alcelaphus buselaphus cokei* Gunther). *Research in Veterinary Science* **15**, 144-146.

Reid, H.W. and Buxton, D. (1984). Malignant Catarrhal Fever of deer. *Proceedings of the Royal Society of Edinburgh B* **82B**, 261-273.

Reid, H.W. and Buxton, D. (1985). Immunity and pathogenesis of Malignant Catarrhal Fever. In "Immunity to Herpesvirus Infections of Domestic Animals" pp117-130. Edited by P.P. Pastoret, E. Thiry and J. Saliki. Brussels: Commission of the European Community.

Reid, H.W., Plowright, W. and Rowe, L.W. (1975). Neutralising antibody to herpesvirus derived from wildebeest and hartebeest in wild animals in East Africa. *Research in Veterinary Science* **18**, 269-273.

Reid, H.W., Buxton, D., Corrigal, W., Hunter, A.R., McMartin, D.A. and Rushton, R. (1979). An outbreak of malignant catarrhal fever in red deer (*Cervus elaphus*). *Veterinary Record* **104**, 120-123.

Reid, H.W., Buxton, D., Pow, I., Finlayson, J. and Berrie, E.L. (1983). A cytotoxic T-lymphocyte line propagated from a rabbit infected with sheep-associated Malignant Catarrhal Fever. *Research in Veterinary Science* **34**, 109-113.

Reid, H.W., Jacoby, R.O., Pow, I. and Buxton, D. (1984a). Culture of large granular lymphocytes from rats with a herpesvirus-associated lymphoproliferative condition. *Immunobiology* **167**, 192.

Reid, H.W., Buxton, D., Berrie, E., Pow, I. and Finlayson, J. (1984b). Malignant catarrhal fever. *Veterinary Record* **114**, 581-583.

Reid, H.W., Buxton, D., Berrie, E., Pow, I. and Finlayson, J. (1985). Culture of malignant catarrhal fever agent. *Biology of Deer Production. The Royal Society of New Zealand Bulletin* **22**, 143-146.

Reid H.W., Buxton, D., Pow, I. and Finlayson, J. (1986). Malignant catarrhal fever: experimental transmission of the "sheep -associated" form of the disease from cattle and deer to cattle, deer, rabbits and hamsters. *Research in Veterinary Science* 41, 76-81.

Reid, H.W., Pow, I. and Buxton, D. (1989a). Antibody to *Alcelaphine herpesvirus* 1 (AHV-1) in hamsters experimentally infected with AHV-1 and the "sheep -associated" agent of malignant catarrhal fever. *Research in Veterinary Science* (in press).

Reid, H.W., Buxton, D., Pow, I. and Finlayson,J. (1989b). Isolation and characterisation of lymphoblastoid cells from cattle and deer affected with "sheep -associated" malignant catarrhal fever. *Research in Veterinary Science* 47, 90-96.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977). Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA Polymerase I. *Journal of Molecular Biology* 113, 237-251.

Rimm, D.L., Pollard, T.D. and Hieter, P. (1988). Resolution of *Acanthamoeba castellanii* chromosomes by pulsed field gel electrophoresis and construction of the initial linkage map. *Chromosoma* (Berl) 97, 219-223.

Roizman, B. (1969). The Herpesviruses- a biochemical definition of the group. *Current Topics in Microbiology and Immunity* 49, 1-79.

Roizman, B. (1982). The Herpesviridae: general description, taxonomy and classification. In "The Herpesviruses" Vol. 1. pp1-23. Edited by B. Roizman. New York and London: Plenum Press.

Rossiter, P.B. (1980). A lack of readily demonstrable virus antigens in the tissues of rabbits and cattle affected with malignant catarrhal fever virus. *British Veterinary Journal* 136, 478-483.

Rossiter, P.B. (1981). Antibodies to Malignant Catarrhal Fever virus in sheep sera. *Journal of Comparative Pathology* 91, 303-311.

Rossiter, P.B. (1982a). Immunoglobulin response of rabbits infected with malignant catarrhal fever virus. *Research in Veterinary Science* **33**, 120-122.

Rossiter, P.B. (1982b). Attempts to protect rabbits against challenge with virulent, cell-associated, Malignant Catarrhal Fever virus. *Veterinary Microbiology* **7**, 419-425.

Rossiter, P.B. (1983). Antibodies to malignant catarrhal fever virus in cattle with non-wildebeest associated malignant catarrhal fever. *Journal of Comparative Pathology* **93**, 93-97.

Rossiter, P.B., Mushi, E.Z. and Plowright, W. (1977). The development of antibodies in rabbits and cattle infected experimentally with an African strain of malignant catarrhal fever virus. *Veterinary Microbiology* **2**, 57-66.

Rossiter, P.B., Gumm, I.D. and Mirangi, P.K. (1988). Immunological relationships between malignant catarrhal fever virus (*Alcelaphine herpesvirus* 1) and bovine cytomegalovirus (*Bovine herpesvirus* 3). *Veterinary Microbiology* **16** 211-218.

Rossiter, P.B., Gumm, I.D., Stagg, D.A., Conrad, P.A., Mukolwe, S., Davies, F.G. and White, H. (1989). Isolation of bovine herpesvirus 3 from African buffaloes (*Syncerus caffer*). *Research in Veterinary Science* **46**, 337-343.

Rurangirwa, F.R., Mushi, E.Z. and Karstad, L. (1982). Malignant catarrhal fever virus specific secretory IgA in nasal secretions of wildebeest calves. *Comparative Immunology of Microbiological Infectious Diseases* **5**, 429-436.

Russell, P.H. (1980). Malignant catarrhal fever in rabbits-reproduction of clinical disease by cell -free virus and partial protection against such disease by vaccination with inactivated virus. *Veterinary Microbiology* **5**, 161-163.

Ruth, G.R., Reed, D.E., Daley, C.A., Vorhies, M.W., Woghlgemuth, K. and Shave, H. (1977). Malignant catarrhal fever in Bison. *Journal of the American Veterinary Medical Association* 171, 913-917.

Rweyemamu, M.M., Karstad, L, Mushi, E.Z., Otema, J.C., Jessett, D.M., Rowe, L., Drevemo, S. and Grootenhuis, J.G. (1974). Malignant catarrhal fever virus in nasal secretions of wildebeest: a probable mechanism for virus transmission. *Journal of Wildlife Diseases* 10, 478-486.

Rweyemamu, M.M., Mushi, E.Z., Rowe, L. and Karstad, L. (1976). Persistent infection of cattle with the herpesvirus of malignant catarrhal fever and observations on the pathogenesis of the disease. *British Veterinary Journal* 132, 393-400.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science* 230, 1350-1354.

Saito, J.K., Gribble, D.H., Berrios, P.E., Knight, H.D. and McKercher, D.G. (1974). A new herpesvirus from goats: preliminary report. *American Journal of Veterinary Research* 35, 847-848.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences* 74, 5463-5467.

Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *Journal of Molecular Biology* 143, 161-178.

Schildkraut, C.L., Marmur, J. and Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *Journal of Molecular Biology* 4, 430-443.

Schirm, S., Muller, I., Desrosiers, R.C. and Fleckenstein, B. (1984). *Herpesvirus saimiri* DNA in a lymphoid cell line established by in vitro transformation. *Journal of Virology* 49, 938-946.

Scott, F.M.M. (1984). Studies on sheep herpesvirus and its role in *Jaagsiekte* (sheep pulmonary adenomatosis). Thesis submitted for the degree of PhD., Council for National Academic Awards.

Seal, B.S., Klieforth, R.B. and Heuschele, W.P. (1987). Evidence for variation among malignant catarrhal fever virus isolates. *Proceedings of the 91st Annual Meeting of the U.S. Animal Health Association* 527-542.

Seal, B.S., Klieforth, R.B., Welch, W.H. and Heuschele, W.P. (1989). *Alcelaphine herpesviruses* 1 and 2. SDS-PAGE analysis of virion polypeptides, restriction endonuclease analysis of genomic DNA and virus replication restriction in different cell types. *Archives of Virology* **106**, 301-320.

Selman, I.E. (1987). The epidemiology of malignant catarrhal fever. *Veterinary Annual* **27**, 98-102.

Selman, I.E., Wiseman, A., Murray, M. and Wright, N. (1974). A clinico -pathological study of bovine malignant catarrhal fever in Great Britain. *Veterinary Record* **94**, 483-490.

Selman, I.E., Wiseman, A., Wright, N.G. and Murray, M. (1978). Transmission studies with bovine malignant catarrhal fever. *Veterinary Record* **102** 252-257.

Senior, M., Halnan, C.R.E. and Tong, E.H. (1962). An outbreak of malignant catarrh among the Pere David deer. *Veterinary Record* **74**, 932-936.

Shih, L-M., Irving, J.M., Zee, Y.C. and Pritchett, R.F. (1988). Cloning and characterization of a genomic probe for malignant catarrhal fever virus. *American Journal of Veterinary Research* **49**, 1665-1668.

Smith, G.E. and Summers, M.D. (1980). The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl -paper. *Analytical Biochemistry* **109**, 123-129.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-517.

Southern, E.M., Anand, R., Brown, W.R.A. and Fletcher, D.S. (1987). A model for the separation of large DNA molecules by crossed field gel electrophoresis. *Nucleic Acids Research* **15**, 5925-5943.

Stamminger, T., Honess, R.W., Young, D.F., Bodemer, W., Blair, E.D. and Fleckenstein, B. (1987). Organization of terminal reiterations in the virion DNA of *Herpesvirus saimiri*. *Journal of General Virology* **68**, 1049-1066.

Storz, J., Okuna, N., McChesney, A.E. and Pierson, R.E. (1976). Virologic studies on cattle with naturally occurring and experimentally induced Malignant Catarrhal Fever. *American Journal of Veterinary Research* **37**, 875-878.

Storz, J., Ehlers, B., Todd, W.J. and Ludwig, H. (1984). Bovine cytomegaloviruses: identification and differential properties. *Journal of General Virology* **65**, 697-706.

Szomolanyi, E., Medveczky, P. and Mulder, C. (1987). *In vitro* immortalization of marmoset cells with three subgroups of *Herpesvirus saimiri*. *Journal of Virology* **61**, 3485-3490.

Thuring, R.W.J., Sanders, J.P.M. and Borst, P. (1975). A freeze-squeeze method for recovering long DNA from agarose gels. *Analytical Biochemistry* **66**, 213-220.

Tong, E.H., Senior, M. and Halman, C.R.E. (1961). An outbreak of malignant catarrh among the Pere David deer. *Proceedings of the Zoological Society of London* **136**, 477-483.

Van den Berg, F.M., Jiwa, M., Rook, R. and Geelen, J.L.M.C. (1988). Analysis and isolation of Cytomegalovirus DNA by field inversion gel electrophoresis. *Journal of General Virology* **69**, 699-704.

Werner, F-J., Bornkamm, G.W. and Fleckenstein, B. (1977). Episomal viral DNA in a *Herpesvirus saimiri*-transformed lymphoid cell line. *Journal of Virology* **22**, 794-803.

Werner, F-J., Desrosiers, R.C., Mulder, C., Bornkamm, G.W. and Fleckenstein, B. (1978). Physical mapping of viral episomes in *Herpesvirus saimiri* transformed lymphoid cells. *ICN-ICLA Symposium on Molecular and Cellular Biology*. Academic Press: New York.

Westbury, H.A. and Denholm, L.J. (1982). Malignant catarrhal fever in farmed Rusa Deer (*Cervus timorensis*). 2. Animal transmission and virological studies. *Australian Veterinary Journal* **58**, 88-92.

Whitenack, D.L., Castro, A.E. and Kocan, A.A. (1981). Experimental malignant catarrhal fever (African form) in white-tailed deer. *Journal of Wildlife Diseases* **17**, 443-451.

Whittaker, P.A., Campbell, A.J.B., Southern, E.M. and Murray, N.E. (1988). Enhanced recovery and restriction mapping of DNA fragments cloned in a new lambda vector. *Nucleic Acids Research* **16**, 6725-6735.

Whitton, J.L., Hundley, F., O'Donnell, B. and Desselberger, U. (1983). Silver staining of nucleic acids. Applications in virus research and in diagnostic virology. *Journal of Virological Methods* **7**, 185-198.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

Young, R.A. and Davis, R.W. (1983). Efficient isolation of genes by using antibody probes. *Proceedings of the National Academy of Sciences* **80**, 1194-1198.

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The *in situ* hybridisation experiments were performed in collaboration with Mr Stephen Maley, Mrs Kate Thomson, Dr Ranald Munro and Dr David Buxton of the Pathology Department and Ms Irene Pow of the Microbiology Department of the MRI. The animal work was performed by Dr Ranald Munro and by my supervisor, Dr Hugh Reid. The infectivity measurements associated with these experiments were performed by Dr Hugh Reid. My role in these experiments was the introduction of this technique to the institute, part of the experimental design, all the probe preparation, hybridisations and subsequent washing and all the analysis.

Appendix 2: Estimation of the copy number of the WC11 repeat sequences by densitometry.

1) Fig. 2.2.1b (C500 *Hin*dII)
The ratio repeat: unique DNA
ie. peak 3: average of peaks 1 and 2
= 19.4: 1

2) Fig. 2.2.1c (WC11 *Sac*I)
The ratio 1800 bp: 1050 bp: 950 bp : unique DNA
ie. peak 1: peak 5 : peak 6 : mean of peaks 1, 2 and 5
= 15.3: 4.4 : 3.6 : 1

3) Fig. 2.2.1d (C500 *Sac*I)
The ratio repeat: unique DNA
ie. peak 3: average of peaks 1, 2 and 5
(peak 4 represents a doublet)
= 18.3: 1

4) C500 *Hin*dII (another gel, not illustrated)
The ratio repeat: unique DNA
= 22.1 :1

5) WC11 *Hin*dII (not illustrated)
The ratio 1800 bp: 1050 bp: 950 bp: unique DNA
= 13.4: 7.9 : 17.8 : 1

6) WC11 *Sma*I (not illustrated)
The ratio 525 bp: 430 bp: 360 bp: 285/275 bp: 165 bp
= 15.3 : 7.8 : 5.5 : 20.5 : 18.3

The above are approximate calculations, with no allowance being made for the different molecular weights of the fragments being compared.

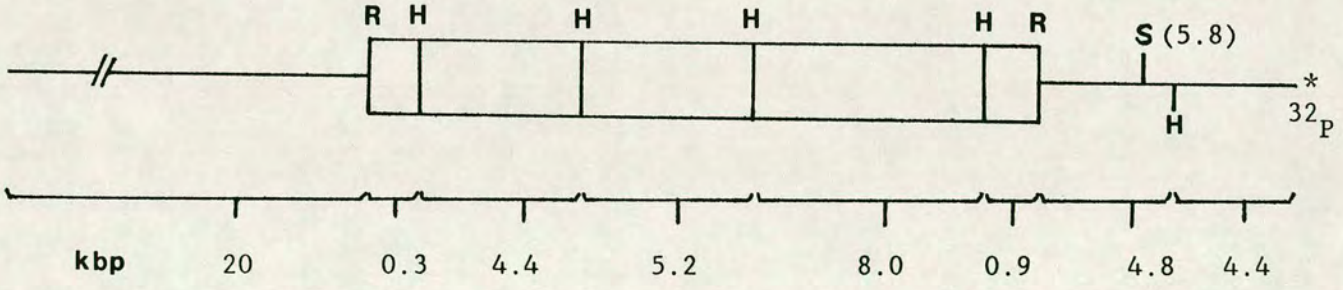
Appendix 3.

Derivation of a restriction map for R65 by partial digestion and cos-end labelling.

The restriction map for R65 was determined by partial digestion and cos-end labelling because this could not be resolved by single and double restriction digestion; the position of the *Sma*I sites were such that they did not define the order and orientation of the R65 subclones. The map was confirmed by the hybridisation properties of the R65 subclones at a later date.

Comparison of the partial *Hin*dIII and *Sma*I profiles illustrated in Fig. 3.1.2 indicates that the subclones of R65 must be ordered 5′-p6f-p6a-H10-3′ and not 5′-p6f-H10-p6a-3′ since the *Sma*I sites of the insert DNA are located in the *Hin*dIII fragments between the second and third and between the fourth and fifth *Hin*dIII sites from the 3′ end of R65 and because p6a, p6f and H10 are known to contain 0, 2 and 3 *Sma*I sites respectively while p6f is known to be located at the 5′ end of the insert. This is illustrated in the figure representing R65, below.

H = *Hin*dIII, R = *Eco*RI, S = *Sma*I (only the *Sma*I site in the 3′ vector arm is shown, for simplicity).



5	4	3	2	1	*Hin*dIII fragment sizes (kbp)
23.3 + 4.4 =27.7	18.1 + 5.2 =23.3	10.1 + 8.0 = 18.1	4.4 + 4.8 +.9 = 10.1	4.4	expected
25.5	22	17	9.7	4.2	observed

Additionally, the *Sma*I fragments of H10 (see Fig. 3.1.1) can be orientated:

5′ - (0.3) - 0.7 - 2.0 - (5) - 3′

from the *Sma*I partial digestion fragments, where 0.7 and 2.0 represent the sizes (kbp) of the internal *Sma*I fragments of H10/R65 while 0.3 and 5 represent the additional *Hin*dIII/*Sma*I fragments of H10/R65.

4	3	2	1	*Sma*I fragment sizes (kbp)
17.3 + .7	15.1 + 2	5.8 + 3.4 + .9	5.8	expected
=18	=17.3	+ 5 = 15.1		(.3-.7-2-5)
11.1 + 2	10.4 + .7	5.8 + 3.4 + .9	5.8	expected
=13.1	=11.1	+ .3 = 10.4		(5-2-.7-.3)
17.5	17	15	5.8	observed

Appendix 4: Size estimation of the WC11 genome by electron microscopy.

The columns show the sizes (kbp) of individual WC11 DNA molecules. In all cases, DNA was prepared by SDS -proteinase K extraction in TNE buffer.

A: WC11 DNA was concentrated by ethanol precipitation.

B: WC11 DNA was concentrated using a rotary evaporator.

C: WC11 DNA was prepared using a rotary evaporator. The DNA was then partially denatured in sodium hydroxide.

A	B	C
143.8	187.6	159.3
110.3	183.3	127.8
96.3	177.0	118.2
78.9	138.3	116.9
77.5	132.4	78.3
71.3	129.3	75.6
70.0	113.6	74.0
67.2	113.5	73.4
64.4	105.8	72.8
62.1	105.4	70.3
61.2	99.7	69.7
58.2	98.9	68.3
49.8	76.9	67.0
43.6	74.9	65.5
39.3		56.6
38.0		56.2
		53.6
		53.3
		48.7
		48.2
		47.2
		42.0
		39.4
		38.2
		37.1
		37.0
		36.2
		34.8
		34.1
		32.8
		32.7
		31.8

Appendix 5

Estimation of the viral copy number in the lymphoblastoid cell line MF/629.

The lymphoblastoid cell line MF/629 was derived from the CSF of a SA-MCF -affected cow.

The amount of DNA per nucleus was taken as 7.3 pg. This figure is the mean of the figures estimated for the cat (6.8 pg) and man (7.8 pg) (Lee *et al.*, 1984).

Assuming the size of the AHV-1 genome to be 160 kbp, the mass of one molecule of AHV-1 DNA is approximately:

$$\frac{660 \times 160{,}000}{6.02\ 10^{23}}\ g$$

$$= 1.75\ 10^{-16}\ g$$

$$= 1.75\ 10^{-4}\ pg$$

Thus, at 1 copy per cell, the ratio of viral DNA: host DNA

$$= 1.75\ 10^{-4} : 7.3$$

$$= 1 : 4.17\ 10^{4}$$

When screening the MF/629 genomic library, 12 clones were obtained with homology to the WC11 clone out of 50,000 clones. This is a ratio of approximately 1: 4200. Comparison with the figure above suggests that there were 10 viral genomes per cell.

However, the WC11 clone hybridised to 3 fragments contained within MF/629 DNA. Based on the assumption that *Hin*dIII cuts with equal frequency in MF/629 and AHV-1 DNA, this alters the expected proportion of viral recombinants from the genomic library to $1 : 1.4\ 10^{4}$ and suggests that there were only 3 viral genome copies per MF/629 cell.

Appendix 6: Published Paper.

Preliminary Characterization of the Alcelaphine Herpesvirus 1 Genome.

Anne Bridgen, Alan J. Herring, Neil F. Inglis and Hugh W. Reid

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Preliminary Characterization of the Alcelaphine Herpesvirus 1 Genome

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SUMMARY

Alcelaphine herpesvirus type 1 (AHV-1) is a causative agent of the fatal lymphoproliferative disease malignant catarrhal fever in deer and cattle. The genomes of the attenuated WC11 isolate and the virulent C500 isolate have been studied. The genome of WC11 comprises a region of unique DNA of approximately 130 kbp, which has a G + C content of 50%, and approximately 30 kbp of additional tandem direct repeat sequences with a G + C content of 72%. WC11 possesses a major repeat sequence of 950 bp interspersed with a small number of related sequences of different length; these sequences are probably terminal in location. DNA from the C500 isolate has a similar restriction profile to that of WC11 in the unique region, but only one repeat sequence of 1050 bp is present. We propose, on the basis of biological and structural properties, that AHV-1 be included within the γ_2 group of herpesviruses of which herpesvirus ateles is the prototype.

INTRODUCTION

Alcelaphine herpesvirus type 1 (AHV-1) is a herpesvirus of the blue wildebeest, *Connochaetes taurinus*, which is an inapparent carrier of the virus. In free-living animals the virus spreads through ocular and nasal secretions (Mushi *et al.*, 1980) such that all wildebeest calves over the age of 7 months are infected (Plowright, 1967). Infection of ruminants other than wildebeest leads to a fatal lymphoproliferative and degenerative disease known as malignant catarrhal fever (MCF) (Plowright *et al.*, 1960; Plowright, 1968, 1982). Economic losses in Africa and in zoological parks can be severe. The disease is a world-wide phenomenon, which indicates that another agent must exist. Epidemiological evidence implicates sheep as a second carrier of infection. However, attempts to isolate a virus capable of inducing MCF from sheep or sheep agent-affected animals have failed repeatedly (Storz, 1976). Little characterization of AHV-1 has been reported to date, and we provide the first account of the genomic organization of this virus.

METHODS

Viral isolates. Experiments were generally performed using the attenuated isolate WC11 (Plowright *et al.*, 1963) which produces cell-free virus. Two varieties of the virulent C500 isolate described by Plowright *et al.* (1975) were employed for comparative purposes: cell-associated C500, C500 ca, and a cell-free derivative, C500 cf.

Preparation of virion DNA. Virus particles were prepared as described by Herring *et al.* (1989). DNA was extracted by incubation in SDS–proteinase K followed by phenol and chloroform extractions. Viral DNA was concentrated by ethanol precipitation using mussel glycogen (Boehringer) as carrier.

Preparation of viral DNA from infected cells. In the single experiment in which viral DNA was prepared from the C500 ca virus isolate, susceptible cells (bovine kidney) were cocultivated with cells from the lymph nodes of a C500 ca-infected rabbit. Following the onset of c.p.e. cells were harvested, washed in phosphate-buffered saline and lysed by resuspension in 0·5% (v/v) NP40 in RSB (RSB is 10 mM-NaCl, 1 mM-$MgCl_2$, 10 mM-Tris–HCl pH 7·5) using gentle homogenization. Chromatin and cellular debris were removed by centrifugation for 10 min at 800 *g*

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and 4 °C. The supernatant fluid was layered over 25% (w/v) sucrose in RSB and viral nucleocapsids were pelleted by centrifugation at 114000 *g* for 30 min at 4 °C. Viral DNA was then prepared from the pellet using the selective extraction procedure of Hirt (1967). The supernatant fraction from this extraction was digested with proteinase K and phenol-extracted as described.

Source of other viral DNAs. We thank R. W. Honess for herpesvirus saimiri (HVS) DNA and clones, P. Medveczky for herpesvirus sylvilagus clones, R. Jarrett for DNA from cells infected with human herpesvirus 6 (HHV-6), J. Arrand for cloned Epstein–Barr virus (EBV) *Bam*HI/W DNA and E. Thiry for bovine herpesvirus 4 (BHV-4) DNA.

Agarose gel electrophoresis. Electrophoresis was performed using gel strengths of 0·4 to 2% (w/v) agarose and TBE buffer (1 × TBE is 134 mM-Tris base, 44 mM-boric acid, 2·6 mM-EDTA). Fragments were transferred to nitrocellulose (Schleicher & Schuell) or nylon (Amersham Hybond) membranes by the bidirectional blotting method of Smith & Summers (1980). DNA fragments of greater than 20 kbp were separated by orthogonal field-alteration gel electrophoresis (OFAGE; Carle & Olson, 1984).

Polyacrylamide gel electrophoresis. DNA fragments of up to 1000 bp were separated by continuous PAGE in Loening's E buffer; fragments of 200 to 4000 bp were separated by discontinuous PAGE in Laemmli's buffer. Gels were prepared according to McClenaghan *et al.* (1984).

Silver staining of polyacrylamide gels. Silver staining provided a sensitive means for the detection of DNA fragments. The method used was a slight modification of that described by Herring *et al.* (1982). Staining with silver nitrate was performed for 20 min only, and sodium borohydride was omitted from the development step. The intensity of stained fragments in polyacrylamide gels was assessed using a Joyce-Loebl Chromoscan 3 densitometer.

Radiolabelling of DNA. DNA was labelled by nick translation (Rigby *et al.*, 1977) or by random hexanucleotide primer extension (Feinberg & Vogelstein, 1983, 1984) using [α-^{32}P]dCTP at 3000 Ci/mmol and 10 mCi/ml.

Hybridization of DNA. Filters were pre-hybridized in 4 × SSC, 5 × Denhardt's solution, 0·1% (w/v) SDS, 200 μg/ml sonicated salmon sperm DNA and 50% (v/v) formamide for at least 30 min at 37 °C, and hybridized in a similar solution with 1 × Denhardt's solution for 12 to 18 h at 37 °C. Filters were rinsed in 4 × SSC, and washed in 50% formamide, 4 × SSC and 1 × Denhardt's solution for 2 × 2 h at 37 °C, followed by 30 min washes in 1 × SSC and 0·5 × SSC at 37 °C. Hybridization to heterologous DNA was performed as above but at 42 °C, and a series of hybridizations in 20%, 30%, 40% and 50% (v/v) formamide were performed. Filters were rinsed in 4 × SSC, and washed twice for 2 h in 20% formamide, 4 × SSC and 1 × Denhardt's solution before autoradiography.

Caesium chloride density gradient centrifugation. Five ml gradients with an initial density of 1·72 g/ml (57% w/v CsCl) were formed in TE buffer pH 7·5 (TE is 10 mM-Tris–HCl, 1 mM-EDTA). The A_{260} profile was measured by upward displacement of the gradient using an ISCO model 184 density gradient fractionator with a u.v. monitor. Fractions of 0·2 ml were collected and the density was determined by refractometry.

Molecular cloning of AHV-1. Two AHV-1 clones were used in the experiments described in this paper. R65 consists of an 18 kbp WC11 fragment cloned into λEMBL4. λEMBL4 DNA was restricted with *Eco*RI and then with *Bam*HI to digest the central fragment of the vector. The small *Eco*RI–*Bam*HI fragments were removed by isopropanol precipitation, and the vector arms were ligated to *Eco*RI-restricted AHV-1 DNA. Phage were recovered by packaging, and recombinant molecules were identified by hybridization to whole viral DNA.

M30 comprises the WC11 950 bp major repeat sequence cloned into M13mp18. WC11 *Hin*dII fragments were separated by agarose gel electrophoresis. The 950 bp sequence was isolated and purified by the freeze–squeeze method of Thuring *et al.* (1975), then ligated to *Sma*I-restricted M13mp18 replicative form DNA.

Preparation of lambda phage. Packaging of lambda DNA was performed according to the method of Grosveld *et al.* (1981). Packaged recombinant EMBL phage were chosen by their ability to plate on P2 lysogens (Kaiser & Murray, 1985). Phage liquid lysates were prepared as described by Murray *et al.* (1977), and phage were purified on a CsCl step gradient. Phage DNA was isolated by phenol extraction.

Preparation of M13 phage. M13 phage were propagated in host strain NM522. Growth and selection of phage were as described by Messing (1983). Replicative form DNA was prepared as described by Maniatis *et al.* (1982).

RESULTS

Base composition of the genome

Early studies of AHV-1 included preparative CsCl density gradient centrifugation of viral DNA performed as the last stage of genome purification. The absorbance profiles of the gradients revealed a major peak at a density of 1·709 and a minor but reproducible satellite component at a density of 1·730 g/ml (Fig. 1). These densities correspond to G + C contents of 50% and 72% respectively (Schildkraut *et al.*, 1962). Such base sequence heterogeneity is typical of gammaherpesviruses. Subsequent restriction analysis has confirmed that the dense satellite peak comprises repeat sequences (discussed below).

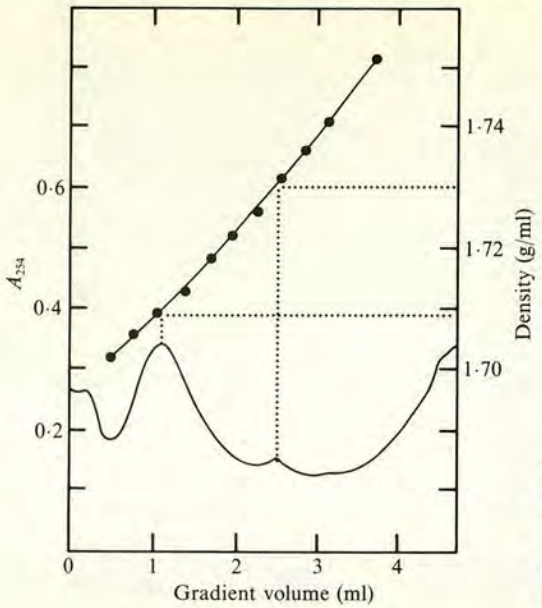

Fig. 1. Caesium chloride density centrifugation of AHV-1 DNA (WC11 isolate). Twenty μg DNA was loaded on a 5 ml 57% (w/v) gradient. The density measurements are shown by filled circles, the line without symbols shows the A_{254}.

Restriction endonuclease analysis

Digestion of the AHV-1 genome with restriction endonucleases has been used to compare isolates of the virus, and to analyse the genome structure.

Comparison of the profiles produced by restriction endonuclease digestion of the DNA from the three available isolates was hindered by the difficulty of preparing sufficient DNA for agarose gel electrophoresis. This has necessitated the use of polyacrylamide gels and silver staining. Comparisons have been made using *Hin*dIII (data not shown), *Hin*dII and *Sac*I (Fig. 2*a*); the results showed that C500 ca and cf are essentially identical (not shown) and that the genome of WC11 differs only slightly from these, particularly in the repeated sequences (discussed below).

Genomic DNA from the WC11 isolate has been analysed using a number of restriction endonucleases. Several enzymes produced supermolar fragments such as those shown in Fig. 3(*a*) and (*b*) for *Sma*I. Similar supermolar fragments were seen using *Alu*I, *Apa*I, *Ava*I, *Hae*III, *Hha*I, *Hin*dII, *Hpa*II, *Msp*I and *Sac*I. No such fragments were seen following digestion of WC11 DNA with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sal*I or *Xho*I. Supermolar fragments are a feature of genomes containing repeated sequences.

The supermolar fragments of C500 are much simpler than those of WC11. *Mbo*I, *Hin*dII and *Sac*I all produced a single supermolar fragment with C500 cf and ca DNA (Fig. 2 and unpublished results). In contrast, *Hin*dII and *Sac*I produced three clearly supermolar fragments with WC11 DNA, and these occurred at different levels of abundance, that is at non-molar ratios (Fig. 2*a*). The fragments shown in Fig. 2(*a*) were separated by PAGE using a discontinuous buffer system. This system maximizes fragment resolution, but has been shown to give aberrant size estimates. More reliable estimates were provided by agarose gel electrophoresis and continuous PAGE, which indicated a size of 1050 bp for the single supermolar fragment of C500 cf, and 1800, 1050 and 950 bp for the three WC11 supermolar fragments. This anomaly in size estimation probably results from an unusually high G+C content of the supermolar fragments.

The difference in abundance of the different WC11 repeat sequences can also be seen in Fig. 3(*b*), which shows three major repeat *Sma*I fragments with mobilities corresponding to 165, 275 and 525 bp, and minor fragments with mobilities of 285, 360 and 430 bp. The 950 bp *Hin*dII repeat sequence from WC11 has been cloned into M13mp18 (clone M30); hybridization

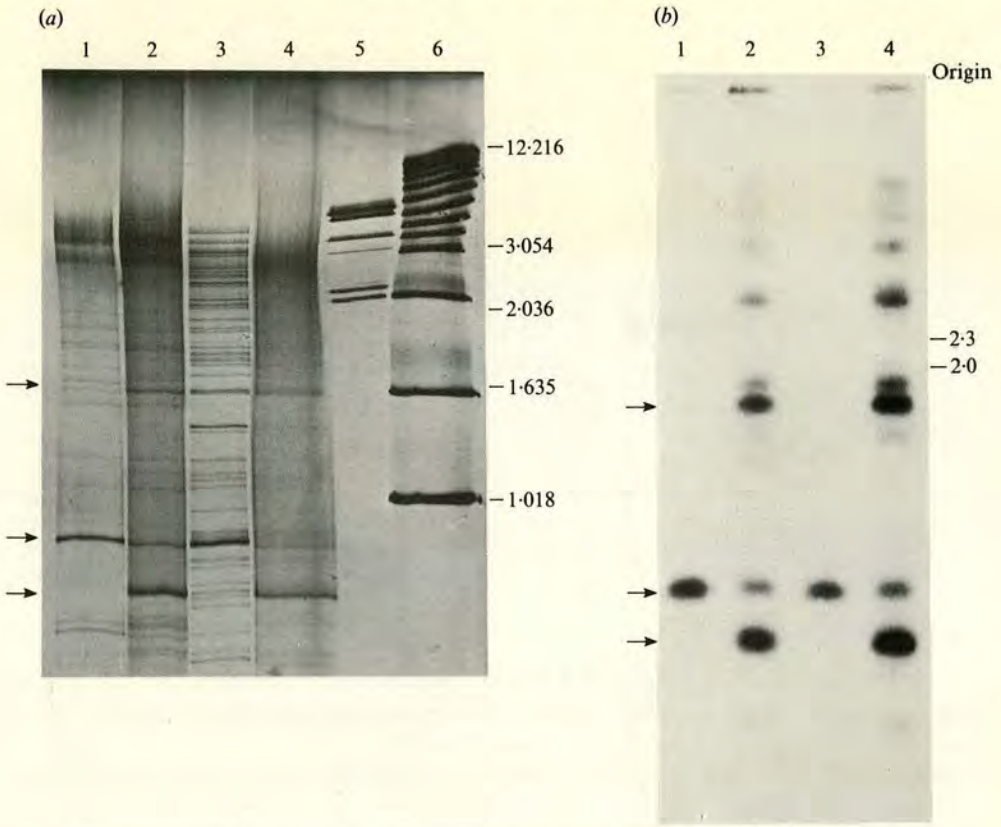

Fig. 2. Supermolar fragments of WC11 and C500 cf isolates produced by *Sac*I or *Hin*dII digestion. Lane 1, C500 cf *Sac*I; lane 2, WC11 *Sac*I; lane 3, C500 cf *Hin*dII; lane 4, WC11 *Hin*dII; lane 5, λcI857 *Hin*dIII; lane 6, 1 kbp ladder. The supermolar fragments are identified with arrows. (*a*) Silver-stained discontinuous polyacrylamide gel. (*b*) Autoradiogram showing hybridization of M30 (cloned WC11 950 bp *Hin*dII supermolar fragment) to a Southern blot of WC11 and C500 cf DNA restricted with *Hin*dII or *Sac*I. Fragments were separated on 2% agarose.

experiments using this clone showed that all the supermolar fragments of C500 and WC11 produced by *Hin*dII, *Sac*I or *Sma*I digestion hybridize with the cloned sequence. Restriction analysis of M30 using *Sma*I showed the insert to consist of the 165, 275 and 525 bp fragments as expected (with the *Hin*dII site within the 525 bp fragment). Direct *Sma*I analysis of C500 cf DNA showed the 1050 bp repeat to consist of fragments of 165, 275, 285 and 360 bp (data not shown). The two additional fragments appear as minor fragments in the WC11 repeats (Fig. 3*b*) suggesting that the C500 and WC11 *Hin*dII and *Sac*I 1050 bp fragments are equivalent. The 1800 bp WC11 *Hin*dII fragment has been shown to contain a 430 bp *Sma*I fragment (data not shown), thus accounting for all the minor WC11 *Sma*I fragments.

Evidence that these repeat sequences reside in the genome as tandemly arranged blocks of various lengths was provided by the following observations. Digestion of WC11 and C500 ca DNA with enzymes such as *Hin*dIII or *Xho*I which do not cut in the repeat sequences, followed by hybridization using the repeat sequence probe M30 showed homology to large fragments of indistinct resolution. Hybridization to *Eco*RI digests showed a ladder of fragments with homology to M30 ranging in size from 8 to 20 kbp with a periodicity of 950 bp. A complex ladder was revealed when WC11 DNA was digested with *Mbo*I and probed with M30 (Fig. 4*a*). This figure resolves at least 15 sets of fragments with a periodicity of 950 bp. Above 3 kbp the

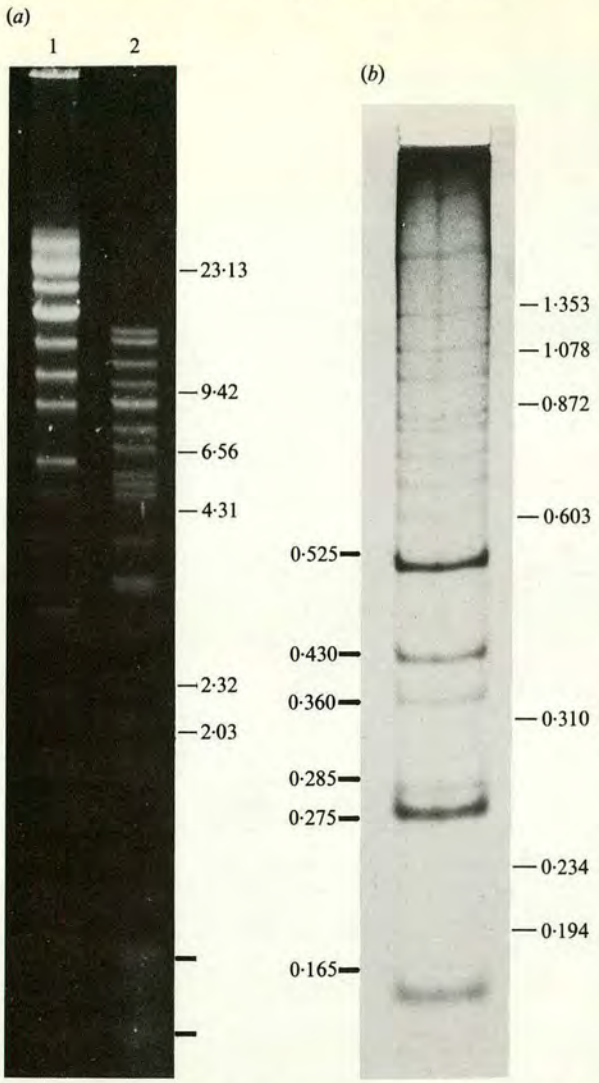

Fig. 3. Electrophoresis of *Xho*I- and *Sma*I-digested WC11 DNA. (*a*) Agarose gel (0·4%) electrophoresis. Lane 1, WC11 *Xho*I; lane 2, WC11 *Sma*I. (*b*) Continuous acrylamide gel (7·5%) electrophoresis showing WC11 *Sma*I fragments visualized by silver staining. The sizes of the fragments and the lambda ladder are shown in kbp.

fragments became increasingly less resolved as their size increases; between 3 kbp and the 1050 bp repeat fragment the ladder was well resolved.

A complex ladder of well defined fragments was observed when WC11 DNA was digested with *Hin*dII or *Sac*I and probed with M30 (Fig. 2*b* and 4*b*). The *Mbo*I ladder was also obtained on restriction of the $\rho = 1{\cdot}730$ fraction from the CsCl density gradient and from digestion of the indistinct large *Hin*dIII fragments of WC11 isolated from an agarose gel (data not shown).

Digestion patterns using the restriction endonucleases *Msp*I and *Hpa*II were identical, indicating that there is no significant methylation of cytosine at CpG dinucleotides.

No single restriction endonuclease has been found to give a profile suitable for genomic size estimation. The most informative profiles are provided by *Eco*RI, *Xho*I and *Sma*I. Restriction

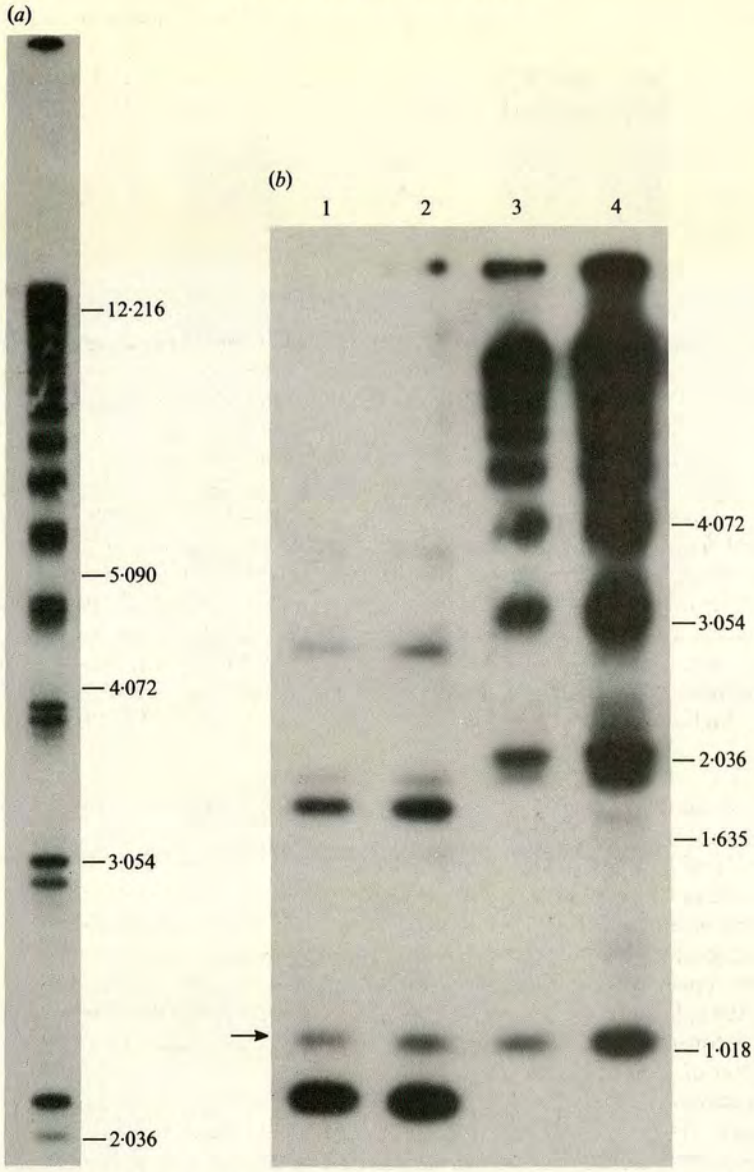

Fig. 4. *Mbo*I repeat ladders. (*a*) Autoradiogram showing WC11 DNA digested with *Mbo*I, separated on 1% agarose and probed with the repeat sequence clone M30. (*b*) Autoradiogram showing WC11 DNA digested with *Sac*I (lane 1), *Hin*dII (lane 2) or *Mbo*I (lanes 3 and 4), separated on 1·2% agarose and probed with M30. The 1050 bp repeat sequence common to both isolates is arrowed.

digestion of WC11 DNA with *Eco*RI produced fragments of 1·5, 3·5, 4·8, 5·2, 23 and two of 50 kbp (with the 50 kbp fragments visualized by OFAGE) in addition to the 8 to 20 kbp variable fragment associated with the repeat DNA. Thus the *Eco*RI fragments summate to approximately 150 kbp. Accurate sizes for the *Sma*I and *Xho*I fragments have not been obtained because of the difficulty of preparing sufficient viral DNA for analysis by agarose gel electrophoresis. The *Sma*I fragments summated to approximately 155 to 165 kbp, depending on the exact number of repeat sequences, and the *Xho*I fragments to approximately 160 kbp.

The number of repeat sequences can be estimated from densitometry or the analysis of the *Mbo*I ladders. Densitometric scanning of silver-stained polyacrylamide gels has yielded estimates of genome copy numbers of 20 to 25 for the single *Sac*I/*Hin*dII repeat of C500. The WC11 isolate produced supermolar fragments at two abundances; the 950 bp *Sac*I/*Hin*dII fragment was present at 15 to 20 copies per genome and the 1800 and 1050 bp *Sac*I/*Hin*dII fragments were each present at 4 or 5 copies per genome.

Taking all these data into account it is likely, therefore, that the genome size is of the order of 160 kbp.

Homology to other herpesviruses

Two AHV-1 DNA clones were used to assess the homology of AHV-1 to a range of herpesviruses. These were M30, the WC11 950 bp *Hin*dII repeat clone, and the unique region clone R65. Southern blot analysis suggests that R65 represents a truncated form of the 23 kbp *Eco*RI fragment. The two AHV-1 clones were used to probe clones of herpesvirus sylvilagus derived from both the unique and repeated regions of the genome, genomic DNA from HVS and BHV-1 (infectious bovine rhinotracheitis virus), HHV-6-infected cellular DNA, cloned *Bam*HI fragment W of EBV, and DNA from a Belgian isolate of BHV-4, V/Test strain.

M30 labelled by nick translation failed to hybridize to EBV *Bam*HI W, but hybridized to a 2·6 kbp *Eco*RI fragment of BHV-4 under conditions of low stringency. R65 labelled by primer extension did not show homology with HHV-6-infected cellular DNA restricted with *Hin*dIII or cloned herpesvirus sylvilagus DNA at low hybridization stringency. There was clear homology at medium stringency of hybridization to the 11·25 kbp *Msp*I C fragment of HVS (data not shown). There was far weaker hybridization to BHV-1 DNA under the same conditions. BHV-1 is an alphaherpesvirus with a G + C content of 71% (Goodheart & Plummer, 1975), which is considerably higher than the G + C content of the unique DNA of HVS.

DISCUSSION

A method of classification of the herpesviruses into alpha, beta and gamma subfamilies on the basis of biological properties has been described by Roizman (1982). Gammaherpesviruses are lymphotropic, and may be further subdivided into B-lymphotropic or γ_1, for example EBV, and T-lymphotropic or γ_2 herpesviruses, for example HVS (Honess, 1984). The γ_2 herpesviruses HVS, herpesvirus ateles, (HVA), herpesvirus aotus type 2 and herpesvirus sylvilagus possess a similar genomic organization, comprising one unique region of DNA bounded by terminal tandem direct repeats of high G + C DNA (Stamminger *et al.*, 1987; Fleckenstein *et al.*, 1978; Fuchs *et al.*, 1985; P. Medveczky, personal communication). In HVS, the total number of repeat sequences is constant, but the number present at either terminus varies from 1 to 33 (Stamminger *et al.*, 1987).

The γ_2 herpesviruses also share numerous biological properties. By definition, all members of the group are T-lymphotropic. Herpesvirus sylvilagus, however, infects both T and B lymphocytes *in vivo* (Kramp *et al.*, 1985). Lymphoblastoid cell lines with the characteristics of large granular lymphocytes have been cultured from animals affected with HVS-induced disease (Ortaldo *et al.*, 1985). Lymphoblastoid cells of this type have also been cultured from rabbits and rats experimentally infected with AHV-1 (Reid *et al.*, 1984; Jacoby *et al.*, 1988), suggesting that these cells may also be important target cells for this virus. It has been suggested that dysfunction of this cell type following infection is responsible for the pathological changes observed in MCF (Reid & Buxton, 1984). Numerous authors have commented upon the similarity of the pathology of AHV-1 to that of the T-lymphotropic gammaherpesviruses (Hunt & Billups, 1979; Patel & Edington, 1980, 1981; Plowright, 1982). The major difference lies in the essentially neoplastic response of animals reacting with HVS or HVA, as compared with the response in MCF-affected animals which is both lymphoproliferative and degenerative. However, rats experimentally infected with AHV-1 develop lesions resembling neoplasia (Jacoby *et al.*, 1988).

Restriction data on the virus have been published in only two reports: Ludwig (1983) and Osorio *et al.* (1985). These papers include restriction profiles for several enzymes, but make no observations concerning genome organization. Osorio *et al.* found marked differences in

Table 1. *Mole percentage G + C**

	Unique DNA	Repeat DNA	Whole genome
Herpesvirus saimiri	35·8	70·6	45·4
Herpesvirus ateles	37·5	74·6	47·1
Herpesvirus aotus type 2	40·2	68·7	48·1
AHV-1	–	72	50

* Adapted from Fleckenstein & Mulder (1980).

Table 2. *Physical properties of γ_2 herpesvirus genomes*

	Genome size (kbp)	Repeat size (bp)	Repeat number
Herpesvirus saimiri	153–161	1444	30–34
Herpesvirus ateles	106	1600	
		1400	
Herpesvirus aotus type 2	151	2700 } related	
		2300 }	
Herpesvirus sylvilagus	108–129	500	15–20
AHV-1 (WC11 isolate)	155–165	1800	4–5
		1050 } related	4–5
		950 }	15–20
AHV-1 (C500 cf isolate)		1050	20–25

* References as in text and Cohrs & Rouhandeh (1987).

restriction profiles between WC11 and a virus, isolated from an Indian gaur, which also causes MCF (Oklahoma strain). It seems likely therefore that WC11 and the Oklahoma virus represent distinct viruses.

The results presented here provide the first molecular evidence for inclusion of AHV-1 within the γ_2 herpesvirus group. The physical properties of the AHV-1 genome are compared with those of the characterized members of this subfamily in Tables 1 and 2, and are shown to be very similar.

Several of the results require further clarification. It cannot be discounted that the virus preparations included defective particles, containing almost entirely high G + C repeat DNA. Such particles have been called H genomes (heavy genomes) and occur with HVS preparations (Fleckenstein *et al.*, 1975). Fig. 1 shows a density profile of WC11 DNA which was not deliberately sheared. It was assumed that a low level of accidental shearing of the DNA gave rise to the small peak corresponding to the high G + C DNA, but the presence of H genomes provides another explanation. It seems very likely that the repeated sequences are terminal as with the other γ_2 herpesviruses. However, attempts to establish the terminal location of the repeated sequences by digestion of WC11 DNA with the exonuclease Bal 31 have consistently failed; this could be explained by the presence of H genomes.

The organization of the repeated sequences in the WC11 isolate is clearly complex. The hybridization results produced with M30 following *Mbo*I, *Hin*dIII and *Sac*I digestion are consistent with the existence of a number of divergent versions of the basic repeat sequence interspersed within blocks of this major 950 bp repeat. This is a similar situation to that observed in herpesvirus aotus type 2 (Fuchs *et al.*, 1985).

Epidemiological evidence indicates that sheep carry an agent capable of inducing MCF in other ungulates. This is supported by immunological evidence (Rossiter, 1981; Herring *et al.*, 1989; H. W. Reid, unpublished results). Preliminary experiments using cloned WC11 unique region DNA as hybridization probes for a related agent in lymphoblastoid cell lines derived from deer and cattle putatively infected with the sheep agent have yielded positive results. We are currently characterizing the homologous sequences.

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REFERENCES

CARLE, G. F. & OLSON, M. V. (1984). Separation of chromosomal DNA molecules from yeast by orthogonal-field-alteration gel electrophoresis. *Nucleic Acids Research* **12**, 5647–5664.

COHRS, R. & ROUHANDEH, H. (1987). Characterization of the genome of *Herpesvirus sylvilagus*. *Intervirology* **28**, 181–184.

FEINBERG, A. P. & VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13.

FEINBERG, A. P. & VOGELSTEIN, B. (1984). Addendum: a technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **137**, 266–267.

FLECKENSTEIN, B. & MULDER, C. (1980). Molecular biological aspects of *Herpesvirus saimiri* and *Herpesvirus ateles*. In *Viral Oncology*, pp. 799–812. Edited by G. Klein. New York: Raven Press.

FLECKENSTEIN, B., BORNKAMM, G. W. & LUDWIG, H. (1975). Repetitive sequences in complete and defective genomes of *Herpesvirus saimiri*. *Journal of Virology* **15**, 398–406.

FLECKENSTEIN, B., BORNKAMM, G. W., MULDER, C., WERNER, F.-J., DANIEL, M. D., FALK, L. A. & DELIUS, H. (1978). *Herpesvirus ateles* DNA and its homology with *Herpesvirus saimiri* nucleic acid. *Journal of Virology* **25**, 361–373.

FUCHS, P. G., RÜGER, R., PFISTER, H. & FLECKENSTEIN, B. (1985). Genome organization of *Herpesvirus aotus* type 2. *Journal of Virology* **53**, 13–18.

GOODHEART, C. R. & PLUMMER, G. (1975). The densities of herpesviral DNAs. *Progress in Medical Virology* **19**, 324–352.

GROSVELD, F. G., DAHL, H. H., DE BOER, E. & FLAVELL, R. A. (1981). Isolation of β-globin-related genes from a human cosmid library. *Gene* **13**, 227–237.

HERRING, A. J., INGLIS, N. F., OJEH, C. K., SNODGRASS, D. R. & MENZIES, J. D. (1982). Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *Journal of Clinical Microbiology* **16**, 473–477.

HERRING, A., REID, H., INGLIS, N. & POW, I. (1989). Immunoblotting analysis of the reaction of wildebeest, sheep and cattle sera with the structural antigens of *Alcelaphine herpesvirus*-1. *Veterinary Microbiology* (in press).

HIRT, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *Journal of Molecular Biology* **26**, 365–369.

HONESS, R. W. (1984). Herpes simplex and 'the herpes complex': diverse observations and a unifying hypothesis. *Journal of General Virology* **65**, 2077–2107.

HUNT, R. D. & BILLUPS, L. H. (1979). Wildebeest-associated malignant catarrhal fever in Africa: a neoplastic disease of cattle caused by an oncogenic herpesvirus? *Comparative Immunology and Microbiology of Infectious Diseases* **2**, 275–283.

JACOBY, R. O., REID, H. W., BUXTON, D. & POW, I. (1988). Transmission of wildebeest-associated and sheep-associated malignant catarrhal fever to hamster, rats and guinea-pigs. *Journal of Comparative Pathology* **98**, 91–98.

KAISER, K. & MURRAY, N. E. (1985). The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries. In *DNA Cloning*, vol. 1, pp. 1–47. Edited by D. M. Glover. Oxford: IRL Press.

KRAMP, W. J., MEDVECZKY, P., MULDER, C., HINZE, H. C. & SULLIVAN, J. L. (1985). *Herpesvirus sylvilagus* infects both B and T lymphocytes *in vivo*. *Journal of Virology* **56**, 60–65.

LUDWIG, H. (1983). Bovine herpesviruses. In *The Herpesviruses*, vol. 2, pp. 135–214. Edited by B. Roizman. New York & London: Plenum Press.

McCLENAGHAN, M., HERRING, A. J. & AITKEN, I. D. (1984). Comparison of *Chlamydia psittaci* isolates by DNA restriction endonuclease analysis. *Infection and Immunity* **45**, 384–394.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory.

MESSING, J. (1983). New M13 vectors for cloning. *Methods in Enzymology* **101**, 20–78.

MURRAY, N. E., BRAMMAR, W. J. & MURRAY, K. (1977). Lambdoid phages that simplify the recovery of in vitro recombinants. *Molecular and General Genetics* **150**, 53–61.

MUSHI, E. Z., KARSTAD, L. & JESSETT, D. M. (1980). Isolation of bovine malignant catarrhal fever virus from ocular and nasal secretions of wildebeest calves. *Research in Veterinary Science* **29**, 168–171.

ORTALDO, J. R., NEUBAUER, R. H., BLANCA, I. & RABIN, H. (1985). Cytotoxic activity of normal and herpesvirus saimiri-transformed nonhuman primate cells. *Cellular Immunology* **95**, 349–357.

OSORIO, F. A., REED, D. E., VAN DER MAATEN, M. J. & METZ, C. A. (1985). Comparison of the herpesviruses of cattle by DNA restriction endonuclease analysis and serologic analysis. *American Journal of Veterinary Research* **10**, 2104–2109.

PATEL, J. R. & EDINGTON, N. (1980). The detection of the herpesvirus of bovine malignant catarrhal fever in rabbit lymphocytes *in vivo* and *in vitro*. *Journal of General Virology* **48**, 437–444.

PATEL, J. R. & EDINGTON, N. (1981). The detection and behaviour of the herpesvirus of malignant catarrhal fever in bovine lymphocytes. *Archives of Virology* **68**, 321–326.

PLOWRIGHT, W. (1967). Malignant catarrhal fever in East Africa. III. Neutralizing antibody in free-living wildebeest. *Research in Veterinary Science* **8**, 129–136.

PLOWRIGHT, W. (1968). Malignant catarrhal fever. *Journal of the American Veterinary Medical Association* **152**, 795–804.

PLOWRIGHT, W. (1982). Malignant catarrhal fever virus: a lymphotropic herpesvirus of ruminants. In *Latent Herpesvirus Infections in Veterinary Medicine*, pp. 279–305. Edited by G. Wittman, R. Gaskell & H. Ryiha. Boston & The Hague: Martinus Nijhoff.

PLOWRIGHT, W., FERRIS, R. D. & SCOTT, G. R. (1960). Blue wildebeest and the aetiological agent of bovine malignant catarrhal fever. *Nature, London* **188**, 1167–1169.

PLOWRIGHT, W., MACADAM, R. F. & ARMSTRONG, J. A. (1963). Growth and characterization of the virus of bovine malignant catarrhal fever in East Africa. *Journal of General Microbiology* **39**, 253–266.

PLOWRIGHT, W., HERNIMAN, K. A. J., JESSETT, D. M., KALUNDA, M. & RAMPTON, C. S. (1975). Immunisation of cattle against the herpesvirus of malignant catarrhal fever; failure of inactivated vaccines with adjuvant. *Research in Veterinary Science* **19**, 159–166.

REID, H. W. & BUXTON, D. (1984). Malignant catarrhal fever of deer. *Proceedings of the Royal Society of Edinburgh* **82B**, 261–273.

REID, H. W., JACOBY, R. O., POW, I. & BUXTON, D. (1984). Culture of large granular lymphocytes from rats with a herpesvirus-associated lymphoproliferative condition. *Immunobiology* **167**, 192.

RIGBY, P. W. J., DIECKMANN, M., RHODES, C. & BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113**, 237–251.

ROIZMAN, B. (1982). The family Herpesviridae: general description, taxonomy and classification. In *The Herpesviruses*, vol. 1, pp. 1–23. Edited by B. Roizman. New York & London: Plenum Press.

ROSSITER, P. B. (1981). Antibodies to malignant catarrhal fever virus in sheep sera. *Journal of Comparative Pathology* **91**, 303–311.

SCHILDKRAUT, C. L., MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *Journal of Molecular Biology* **4**, 430–443.

SMITH, G. E. & SUMMERS, M. D. (1980). The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl paper. *Analytical Biochemistry* **109**, 123–129.

STAMMINGER, T., HONESS, R. W., YOUNG, D. F., BODEMER, W., BLAIR, E. D. & FLECKENSTEIN, B. (1987). Organization of terminal reiterations in the virion DNA of herpesvirus saimiri. *Journal of General Virology* **68**, 1049–1066.

STORZ, J. (1976). Virologic studies on cattle with naturally occurring and experimentally induced malignant catarrhal fever. *American Journal of Veterinary Research* **37**, 875–878.

THURING, R. W. J., SANDERS, J. P. M. & BORST, P. (1975). A freeze-squeeze method for recovering long DNA from agarose gels. *Analytical Biochemistry* **66**, 213–220.

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