Recombinant Surface Glycoproteins of Feline Leukaemia Virus

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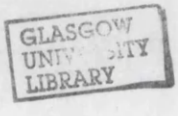

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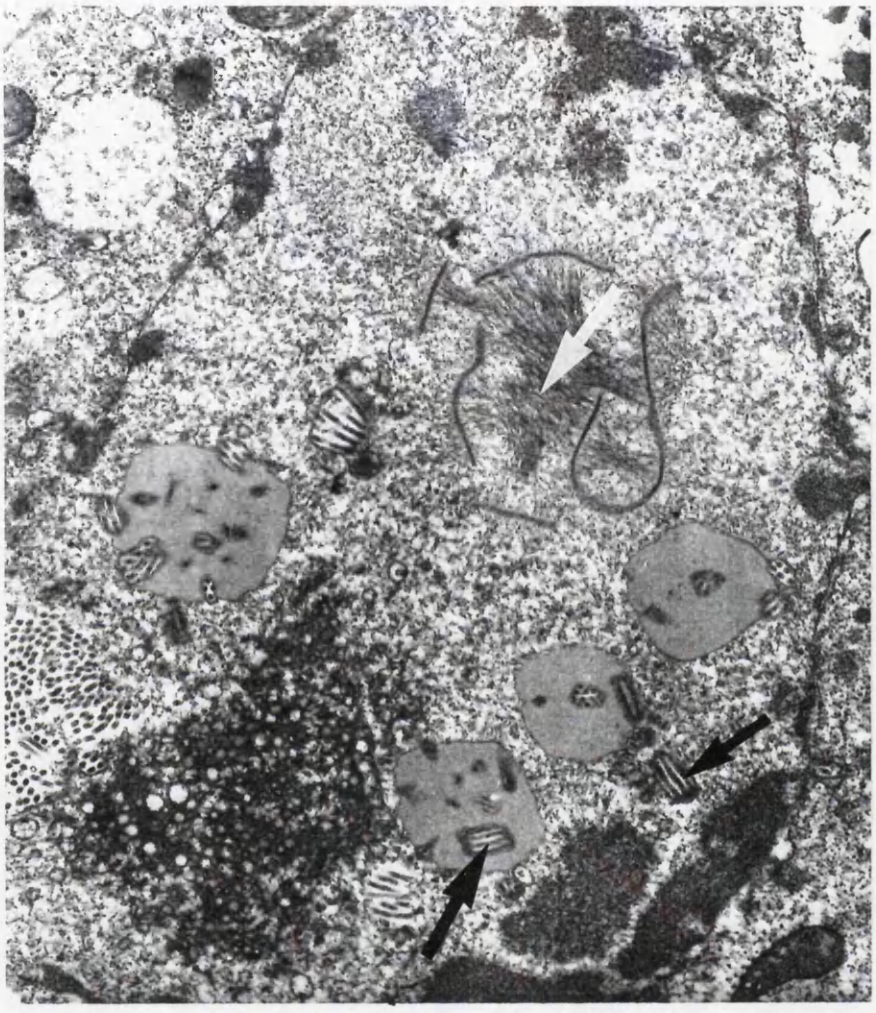
Frontispiece

Electron micrograph of an insect cell infected with wild type *Autographa californica* multiple nuclear polyhedrosis virus .



The black arrows indicate viral particles containing multiple nucleocapsids. The particles are of two types: occluded particles are encased in polyhedral crystals - made of the protein polyhedrin; non-occluded particles are free in the cytoplasm. The fibrillar material (white arrow) is believed to be accumulations of the viral p10 protein.

The baculovirus expression sytem is based upon the replacement of the viral gene encoding for the polyhedrin protein with a foreign gene of interest.

Photograph kindly supplied by Dr Helen Laird, Electron Microscopy Unit, Department of Veterinary Pathology, University of Glasgow.

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The work detailed in this thesis, except where stated, was performed entirely by me and has not been published elsewhere.

Abbreviations

AcMNPV	*Autographa californica* multiple nuclear polyhedrosis virus
ALV	avian leukaemia virus
ATP	adenosine triphosphate
ddNTP	equimolar dideoxyadenosine, dideoxycytidine, dideoxyguanosine and dideoxythymidine triphosphates
DMEM	Dulbecco's modified Eagle's minimal essential medium
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	equimolar deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine triphosphates
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(baminoethyl ether) N N N' N' tetraacetic acid
ELISA	enzyme linked immunoabsorbant assay
FCS	foetal calf serum
FeLV	feline leukaemia virus
FIV	feline immunodeficiency virus
HEPES	N-2-hydroxyethylpiperazine-N'-2-etanesulphonic acid
HIV	human immunodeficiency virus
kbp	kilobase pairs
kDa	kilo-Daltons
M	molar
MAb	monoclonal antibody
ng/μg/mg	nano / micro / milli-grammes
μl/ml	micro / milli-litres

mm	millimetres
mM	millimolar
msec	milliseconds
MuLV	murine leukaemia virus
NP40	Nonidet P40
OD_x	optical density measured at wavelength x nanometres
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RBS	receptor binding site
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Rosewall Park Memorial Institute medium
SDS	sodium dodecyl sulphate
SPF	specific pathogen free
TBS	tris buffered saline
TEMED	N,N,N',N'-tetamethylethylene diamine
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol
UV	ultraviolet
V	volts
VCSR	viral cell surface receptor
v/v	millilitres per 100 millilitres
w/v	milligrammes per 100 millilitres

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Summary

Feline leukaemia virus (FeLV) is a major cause of degenerative and proliferative diseases of myeloid, lymphoid and erythroid origin in domestic cats. The envelope of FeLV, which is a typical retrovirus, is studded with a transmembrane protein (TM) that anchors an external surface glycoprotein (SU). The FeLV SU (gp70) carries both neutralising epitopes and subgroup phenotypic determinants. FeLV occurs naturally in 3 subgroups, which are defined by a superinfection interference assay, suggesting that three FeLV cellular receptors exist, one for each subgroup. The regions of SU which determine the A/C phenotype have been defined as lying in a small variable region towards the amino terminus of the protein.

The aims of this project were to produce recombinant SU's from FeLV subgroups A and C (and two A/C chimeras) and to use them in studies of virus/host interactions. The first interaction that was investigated was that which occurs between FeLV and naturally occuring neutralising antibodies. The second interaction that was examined was that which occurs between FeLV and the surface of the host cell. A subsidiary aim was to use the FeLV-A recombinant SU in vaccine studies.

Recombinant baculovirus vectors containing the coding sequences of the entire FeLV SU together with N terminal components of TM from FeLV-A/Glasgow 1 and FeLV-C/Sarma were constructed. The vectors were co-transfected with baculovirus DNA into insect cells and the resulting recombinant baculoviruses were plaque purified. The recombinant baculoviruses were used to produce glycoproteins, called Bgp70-A and Bgp70-C respectively, which were concentrated by ultrafiltration. The proteins were further concentrated and partially purified by lentil lectin affinity chromatography. Two chimeric FeLV-A/C recombinant proteins (Bgp70-215 and Bgp70-VC) were also produced using the baculovirus expression system. The coding sequences used to produce these proteins were derived from chimeric FeLVs that are classified in superinfection interference assays as belonging to subgroup C. The recombinant viruses were demonstrated to contain the correct coding sequences for the subgroup determining region by a mixture of PCR and restriction enzyme digest analysis.

Several investigations were performed into the biological properties of the proteins. All the Bgp70's have an approximate relative molecular mass of 80 kDa. The proteins are aberrantly glycosylated and are not cleaved. In accordance with the recently published findings of other workers, only small quantities of the proteins are produced. An anti-FeLV SU monoclonal antibody bound to the proteins in both ELISAs and western blots. Furthermore, a recovered cat serum which had been derived from a natural infection with FeLV-A contained antibodies that bound to proteins in western blots. However, this serum did not contain antibodies which bound to Bgp70-C in ELISAs. Studies with subgroup-specific monoclonal

antibodies demonstrated that Bgp70-A, -VC and -215 were indistinguishable. In similar ELISAs both a monoclonal antibody and a recovered cat serum, which were specific for FeLV-C/Sarma, only bound to Bgp70-C.

On the basis of these results, the ability of the recombinant proteins to block the action of neutralising antibodies was examined. It was demonstrated that Bgp70-A, -VC and -215 interfered with the action of neutralising antibodies from recovered cat sera which had been derived from a natural infection with FeLV-A, whereas Bgp70-C did not. These results suggest that the major neutralising epitope(s) of FeLV lies outside the subgroup determining region.

Infection interference assays were performed to investigate the ability of the recombinant proteins to serve as ligands for the FeLV cell surface receptors. It was demonstrated that Bgp70-A interfered with infection by FeLV-A of a susceptible cell line, whereas Bgp70-C, -VC and -215 did not. Conversely, Bgp70-C, -VC and -215 interfered with the infection by FeLV-C/FA27C of a susceptible cell line, but Bgp70-A did not. These data suggest that the Bgp70's may be sufficiently similar to the wild type proteins to serve as ligands with which to identify the FeLV cell surface receptors. Co-immunoprecipitation studies were performed using surface labelled cells. All the proteins bound to several cell surface proteins from both permissive and non-permissive cell lines. There were no differences in the spectrum of cell proteins precipitated between the subgroups.

The potential use of Bgp70-A as an FeLV vaccine was also examined. The protein did not induce detectable prechallenge gp70 antibodies in any of the five vaccinates. This was possibly due to the small quantities used. One of the vaccinates showed a delayed onset of viraemia when given a severe challenge and another vaccinate never became viraemic. In another group, which contained cats vaccinated with a commercial vaccine (Leucogen, Virbac), four out of five cats rapidly became persistently viraemic. The fifth cat became p27 antigenaemic, but never became viraemic. In contrast, all of the unvaccinated controls rapidly became viraemic. One of the controls subsequently recovered from the infection. This result suggests that neither vaccine protected the cats against the challenge.

The experiments described in this thesis demonstrate that recombinant surface glycoproteins of FeLV produced in the baculovirus expression system can be used to study initial virus/host interactions. It is suggested that the ease with which these proteins can be produced, and their isolated coding sequences manipulated, represent a significant improvement on previously reported methods.

Chapter 1. Introduction

This thesis describes studies on feline leukaemia virus (FeLV) and, in particular, on FeLV recombinant surface glycoproteins produced in a baculovirus expression system. The aim of the project was to produce the recombinant glycoproteins and to assess their functional properties in a variety of systems. In particular, the ability of the proteins to act as ligands for virus neutralising antibodies and FeLV cell surface receptors was investigated. A subsidiary aim of the project was to use one of the recombinant glycoproteins in vaccine studies.

FeLV surface glycoproteins are the target of virus neutralising antibodies and the site of the initial virus / host cell interaction (Jarrett *et al.,* 1972; Sarma and Log, 1973; Russell and Jarrett, 1978a; Salerno *et al.,* 1978; Lutz *et al.,* 1980). This chapter contains an introduction to FeLV biology, followed by an overview of recent advances in the analysis of initial virus / host cell interactions. Finally, a description of basic baculoviral biology is presented to facilitate the understanding of the baculovirus expression system.

1.1 Feline Retroviruses

Cats are afflicted with a number of retroviruses, of which two are of considerable importance to clinical veterinary science: FeLV and feline immunodeficiency virus (FIV). Three other feline retroviruses are also known; RD-114 virus, feline syncytium forming virus and endogenous feline leukaemia virus.

1.1.1 Feline Leukaemia Virus

FeLV was discovered in a multi-cat household in Glasgow in which a cluster of lymphomas had occurred (Jarrett *et al.,* 1964a; Jarrett *et al.,* 1964b). The authors of these papers demonstrated that the virus caused disease in experimentally infected cats and, in appearance, resembled murine leukaemia viruses (MuLVs) which were later classified as retroviruses. This breakthrough established that retroviruses, which many researchers had previously believed only caused disease in certain inbred strains of mice and fowl, were pathogenic in outbred mammalian populations. Later it was demonstrated that the virus was horizontally transmitted (Hardy *et al.,* 1973), caused non-neoplastic disease (Anderson *et al.,* 1971; Anderson and Jarrett, 1971) and the virus-transformed cells were the targets of immune surveillance (Essex *et al.,* 1975). These findings caused a resurgence of interest in the study of retroviral biology in outbred populations and ultimately paved the way for the identification of the human retroviruses.

1.1.2 Feline Immunodeficiency Virus

FIV was discovered in 1986 in a multi-cat household with a cluster of unusual infections in California (Pedersen *et al.,* 1987). It has since been shown to be a lentivirus with a worldwide distribution, associated with a variety of chronic infections and T cell depletion (Pedersen *et al.,* 1989; Torten *et al.,* 1991). It is an important model for human immunodeficiency virus (HIV) infection (Neil, 1990; Jarrett *et al.,* 1991). There is thought to be no epidemiological association between FIV and FeLV infection (Hosie *et al.,* 1989). Cats which are already infected with FeLV do, however, show more signs during the initial viraemic phase of experimental FIV infection (Pedersen *et al.,* 1990). Several reviews of clinical aspects of FIV infection and the molecular biology of FIV have been published (Hosie *et al.,* 1989; Sparger *et al.,* 1989).

1.1.3 Other Feline Retroviruses

(1) RD 114

RD-114 is an endogenous feline retrovirus which is related to the baboon endogenous virus (Livingston and Todaro, 1973; Sen *et al.,* 1978). RD-114 was initially identified in human tumour cells which had been injected into a foetal cat and was originally thought to be of human origin (McAllister *et al.,* 1972). The virus is described as xenotropic as it can replicate in the cell lines of several heterologous species, but only in a few feline cell lines (Livingston and Todaro, 1973). It has recently been suggested that the host range restriction of RD-114 is determined by altered cellular receptors and aberrant glycosylation of the *env* gene product in non-permissive cell lines (Dunn *et al.,* 1993).

(2) Feline syncytium forming virus

Feline syncytium forming virus (FeSFV) is a member of the genus of retroviruses called spumaviruses (Riggs *et al.,* 1969). FeSFV is widespread amongst cats but is thought to be non-pathogenic (Jarrett *et al.,* 1974). It acts as an important contaminant in some retroviral assays.

(3) Endogenous feline leukaemia virus

Endogenous feline leukaemia viruses (enFeLVs) were first identified as FeLV-related DNA sequences in cells from an FeLV-free cat colony (Okabe *et al.,* 1976). They are a group (7-20) of defective, mostly full length, FeLV-like proviruses contained within the feline cell. A number of enFeLV sequences have been molecularly cloned (Soe *et al.,* 1983; Soe *et al.,* 1985). They differ from FeLV principally in the U3 region and in a portion of the *env* gene (Stewart *et al.,* 1986).

They are thought to represent an historical record of defective FeLV infection of germ line cells. They are inherited in a Mendelian fashion.

1.2 Feline Leukaemia Virus

1.2.1 Virology

(1) Classification

FeLV is a member of the family *Retroviridae*, which are RNA-containing enveloped viruses. The taxonomic features of the Retroviridae are given in Table 1.1, which was derived from comprehensive reviews of the subject (Teich, 1985; Coffin, 1992). FeLV was formerly classified as an oncovirus (Teich, 1985), but is now regarded as being a member of the mammalian type C retrovirus group (Coffin, 1992). FeLV is believed to have originated several millions of years ago from an ancestral rodent retrovirus (Benveniste *et al.*, 1975).

(2) Viral Structure

FeLV contains two copies of a single stranded, RNA genome, which is approximately 8000 base pairs (bp) long. The genome encodes 3 polyproteins which undergo post-translational modification by endogenous proteolysis. The nucleic acid, with its associated reverse transcriptase (RT) and integrase (IN) enzymes, is contained in a protein core. The protein core is formed by 4 proteins; matrix protein (MA), capsid protein (CA), nucleocapsid protein (NC) and a fourth protein, p12, which is of unknown function. This core is surrounded by a lipid based envelope studded with a transmembrane viral-encoded protein (TM) to which is attached by disulphide bridges the external surface glycoprotein (SU) (Pinter and Fleissner, 1979). TM and SU are also referred to as p15(E) and gp70 respectively.

(a) Replication of FeLV

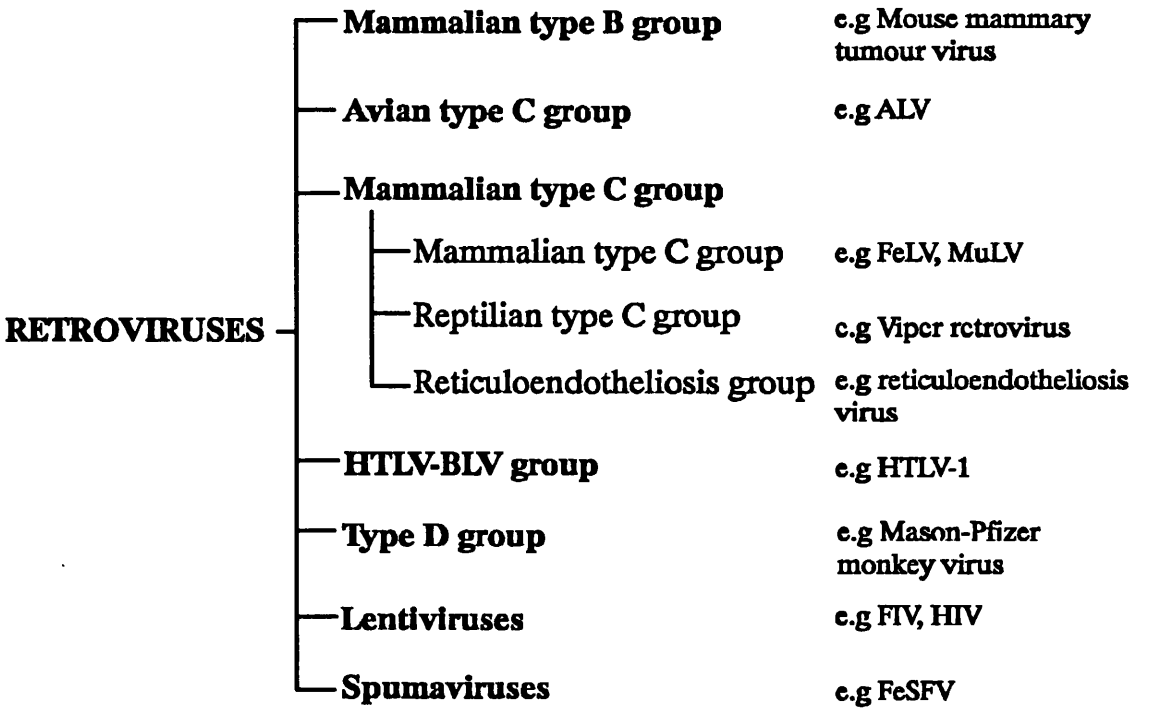
The replication cycle of FeLV has not been studied in detail, but it is generally assumed to follow the same sequence as other retroviruses. On entering each cell the viral RNA is uncoated and reverse transcribed to form a single stranded DNA molecule, which is then converted into a double stranded form, the provirus (Varmus and Swanstrom, 1985). At each end of the RNA genome are found short terminal repeats, called *r*, which contain unique regions called U3 and U5 respectively. The short terminal repeats are important for the synthesis of the proviral DNA. During this process the unique sequences of *r*, U3 and U5, are duplicated at both ends, resulting in the formation of the proviral long terminal repeats (LTRs) which contain both U3 and U5 (Temin, 1981). The double stranded DNA provirus migrates to the nucleus where a proportion becomes circular

Table 1.1 Taxonomy of Retroviridae

a) Taxonomic features

Morphological properties	Spherical enveloped virions with surface projections and containing an icosahedral capsid.
Nucleic Acid	Two identical subunits of linear, positive sense, single-stranded RNA. Capped at 5' end, polyadenylated at 3' end.
Protein	About 60% by weight.
Lipid	About 35% by weight. Derived from host cell membrane.
Carbohydrate	About 4% by weight. Associated with *env* gene products.
Physico-chemical properties	Density 1.16-1.18g /ml in sucrose. Sensitive to lipid solvents, detergents and heat inactivation. Resistant to UV and X irradiation

b) Classification of retroviruses



(Panganiban, 1985). The integrase protein (IN), which may possess an endonuclease activity to cut the circular forms, removes 2 bases from the 3'-hydroxyl termini and mediates the integration of the nicked linear precursor into the host cell genome (Grandgenett and Mumm, 1990). Following integration the provirus is transcribed into positive sense RNA which is then translated or incorporated into budding virions. A spliced messenger RNA is also produced which encodes for the envelope proteins (Papenhausen and Overbaugh, 1992).

The transcriptional products of the provirus and structure of the mature virion are diagrammatically represented in Figure 1.1 which was adapted from published reviews (Hardy, 1980b; Dickson *et al.* 1985).

(3) Natural Infection

FeLV is transmitted in saliva by the oronasal route, presumably by mutual grooming between cats (Hardy *et al.,* 1973). After the virus has entered the host, it replicates first in the lymphocytes of the tonsils and pharyngeal lymph nodes, which then spread the infection to the systemic lymphoid tissues, including the bone marrow. During these early stages gut-associated lymphoid tissue is also infected (Rojko *et al.,* 1979b).

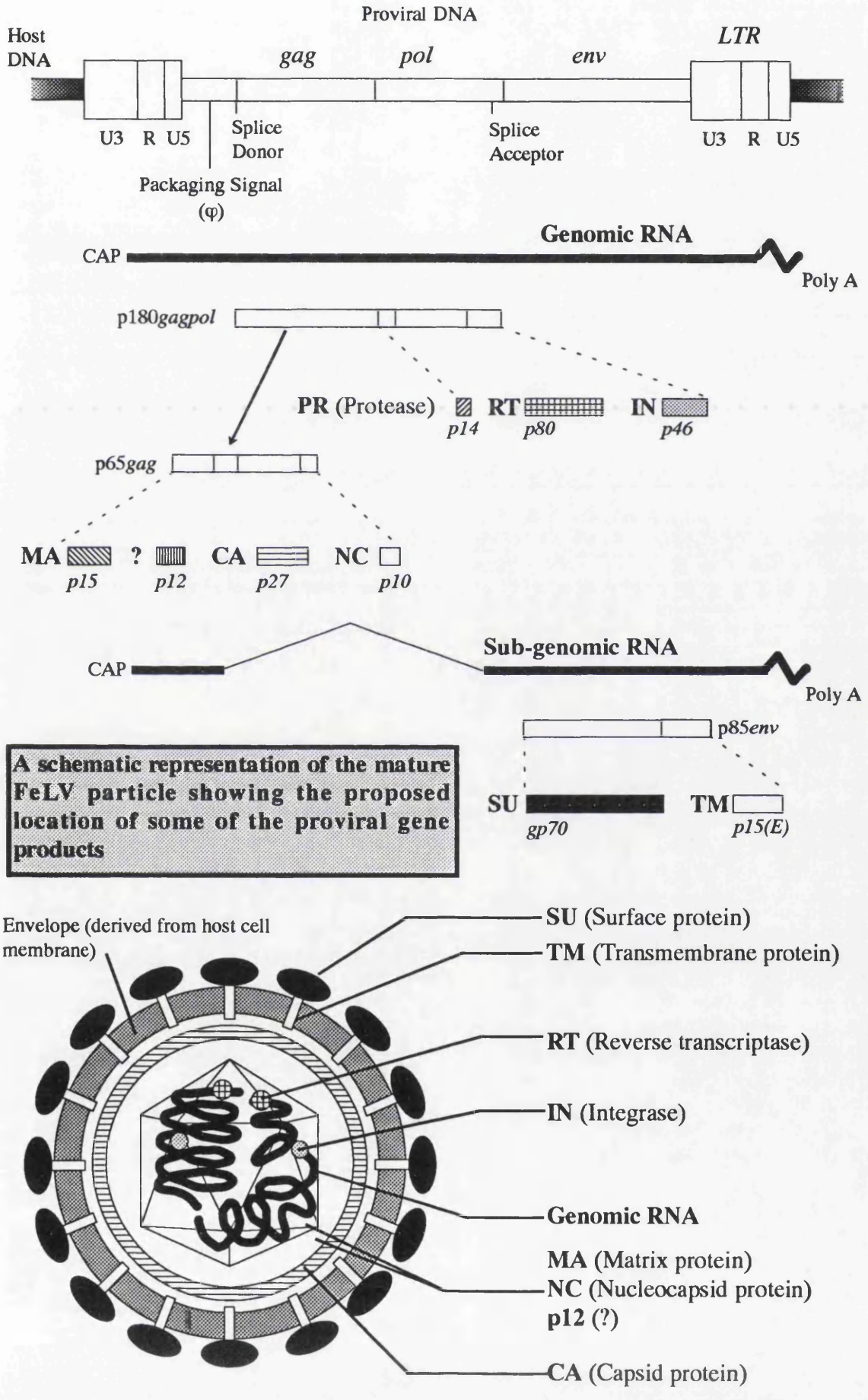
At this stage the cat may eliminate the infection, perhaps due to the production of low titres of virus neutralising antibodies or by other mechanisms not yet elucidated (see below). Alternatively, due to the large numbers of bone marrow cells that are infected the virus may disseminate widely throughout the body. Virus can be found in the plasma or in infected neutrophils and platelets (Rojko *et al.,* 1979b). The appearance of such infected cells in the blood is considered to be the hallmark of persistent viraemia.

As a third alternative, the cat may contain the infection within the bone marrow in a latent state for a variable period of time. Occasionally such latently infected cats may revert to the viraemic state, particularly when given immunosuppressive doses of steroids (Rojko *et al.,* 1982), or enduring physiological stresses such as lactation (Pacitti *et al.,* 1986). In most cats, however, the latent infection appears to be eventually eliminated (Pacitti, 1987).

(4) Subgroups of FeLV

Three subgroups of FeLV (A, B and C) have been defined on the basis of interference with superinfection (Sarma and Log, 1973). These authors demonstrated that cells infected with one isolate of FeLV could not be superinfected with isolates of the same subgroup. Subsequently, subgroup-specific

Figure 1.1 The products and basic structure of the FeLV provirus



A schematic representation of the mature FeLV particle showing the proposed location of some of the proviral gene products

differences in the host range *in vitro* were demonstrated (Jarrett *et al.,* 1973; Sarma *et al.,* 1975). The existence of three FeLV subgroups with different host ranges has traditionally been taken as evidence of the presence of three cellular receptors, one for each subgroup (Sarma and Log, 1973; Jarrett, 1984; Riedel *et al.,* 1986; Donahue *et al.,* 1988; Rigby *et al.,* 1992). Recently however, some doubt has been cast on this assumption (Reinhart *et al.,* 1993).

The evidence for three subgroups was originally augmented by neutralisation data (Sarma and Log, 1973). Subsequently, others (Russell and Jarrett, 1978b) found that a degree of cross-neutralisation occurred between the subgroups.

FeLV subgroup A (FeLV-A) is found in all cases of FeLV infection (Jarrett *et al.,* 1978). It is antigenically monotypic (Russell and Jarrett, 1978b) and is believed to be responsible for inter-host transmission. FeLV-A infects only feline cells (Jarrett *et al.,* 1973; Sarma *et al.,* 1975). A high degree of sequence conservation has been observed between several isolates of FeLV-A that were originally isolated in different countries and several years apart (Donahue *et al.,* 1988). This study suggested that stringent selection against antigenic changes is occurring at the level of inter-host transmission.

FeLV-B is found in approximately 33% of FeLV positive cats that are otherwise healthy: this proportion rises to about 58% when viraemic cats with lymphoma are examined (Jarrett *et al.,* 1978). This result suggested that FeLV-B may be associated with an increased risk of developing neoplastic disease. FeLV-B replicates in wide range of cell lines derived from heterologous species, including human cells (Jarrett *et al.,* 1973), but not guinea pig cells (Sarma *et al.,* 1975). Available sequence data and experimental evidence suggests that FeLV-B arises by recombination with enFeLV (Nunberg *et al.,* 1984b; Stewart *et al.,* 1986; Overbaugh *et al.,* 1988b; Neil *et al.,* 1991). The minimal requirement of enFeLV sequences required to transform FeLV-A into FeLV-B has not been determined. It has been demonstrated that the *in vitro* properties of FeLV can be altered by recombination with enFeLV and that the enFeLVs which encode a full length *env* gene can provide most of the envelope sequences necessary for a replication competent provirus (Pandey *et al.,* 1991). From these studies it is also clear that FeLV-B may continuously evolve *in vivo* by means of novel mutations and recombinations.

FeLV-C isolates are rare; only 2% of all FeLV isolations contain this subgroup (Jarrett *et al.,* 1978). FeLV-C occurs in association with FeLV-A or FeLV-A and FeLV-B (Jarrett *et al.,* 1978). FeLV-C isolates are uniquely associated with the development of pure red cell aplasia (Hoover *et al.,* 1974; Mackey *et al.,* 1975;

Onions *et al.,* 1982). FeLV-C replicates in several cell lines derived from heterologous species, including guinea pig cells (Sarma *et al.,* 1975). Several isolates of FeLV-C are indistinguishable from FeLV-A in neutralisation assays (Russell and Jarrett, 1978b). The prototypic isolate, FeLV-C/Sarma, can be distinguished from FeLV-A. FeLV-C is thought to arise by mutation from FeLV-A (Neil *et al.,* 1991), though FeLV-C/Sarma probably contains additional sequences derived from enFeLV. Subsequently Rigby (1989) sequenced the *env* gene of several other FeLV-C isolates and demonstrated by site directed mutagenesis that the determinants of the superinfection interference phenotype of FeLV-C were located within the VR 1 region (Neil *et al.,* 1991). These results have subsequently been confirmed by others (Brojatsch *et al.,* 1992). The sequences of VR 1 from various FeLV isolates, including two site-directed mutants (Rigby, 1989), are illustrated in Figure 1.2. The disease-causing and infectivity phenotypes are, however, not completely associated with this small region (Rigby *et al.,* 1992). Other sequence differences, possibly near VR 5, may play a role in the generation of these phenotypes (Rigby *et al.,* 1992). This possibility has still to be fully investigated.

1.2.2 Clinical Aspects

(1) Clinical Disease

The diseases caused by, or associated with, FeLV have been extensively reviewed elsewhere (Jarrett, 1985; Hardy, 1980a; Mackey, 1975). The range of clinical conditions associated with FeLV is shown in Table 1.2.

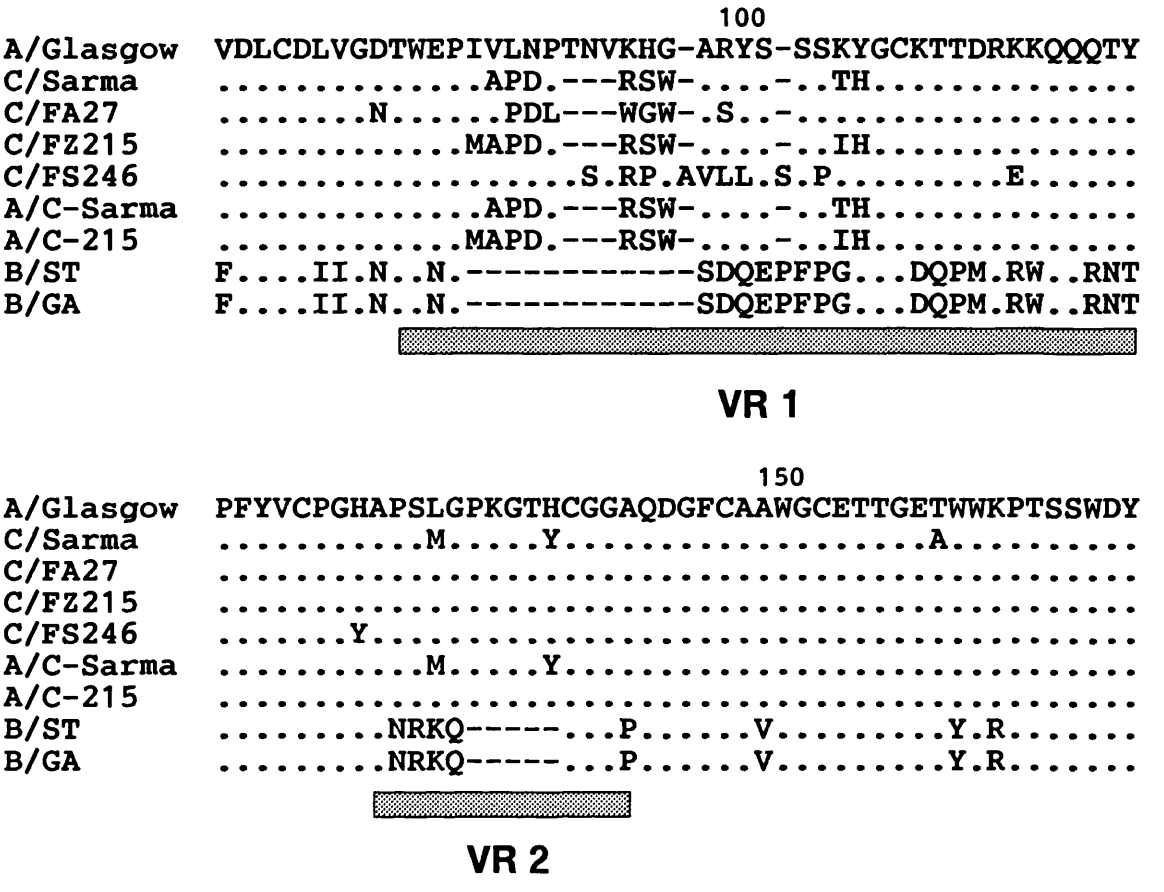
Some clinical conditions have been associated with particular isolates of FeLV, for example, myeloid leukaemia and FeLV-GM-1 (Testa *et al.,* 1988). Often, these isolates are replication defective (Tzavaras *et al.,* 1990). Only one disease, pure red cell aplasia, is associated with a particular subgroup of FeLV, subgroup C.

(a) FeLV induced immunodeficiency

Many oncogenic retroviruses are associated with an initial immune dysfunction shortly after infection. Immunodeficiency may persist for the remainder of the cat's life. Impairment of the correct functioning of the feline immune system is thought to be involved in the pathogenesis of many FeLV associated diseases. Immunodeficiency is one of the leading causes of morbidity and mortality in persistently viraemic cats (Jarrett, 1985).

Immunodeficiency may be due to non-specific viral induced factors such as tumour development or the immunosuppressive properties of certain viral proteins. It has

Figure 1.2 Sequence of various FeLV isolates around VR 1 region



The figure shows the predicted amino acid sequences of various isolates of FeLV around the subgroup-determining region, VR 1. The numbering corresponds to the predicted amino acid residues of the envelope polyprotein precursor of FeLV-A/Glasgow 1 (Stewart *et al.*, 1986). The dots indicate sequence homology: the dashes indicate spaces introduced to conserve alignment. The sequences of the two FeLV-A/C chimeras, FeLV-A/C-Sarma and FeLV-A/C-215, used in this project are also shown. The VR 1 and VR 2 regions are indicated and are derived from Neil *et al.*, (1991).

The sequences in this diagram were derived from the following sources; FeLV-A/Glasgow-1 (Stewart *et al.*, 1986); FeLV-C/Sarma (Reidel *et al.*, 1986); FeLV-B/Gardner-Arnstein (Elder and Mullins, 1983); FeLV-B/Snyder-Theilen (Nunberg *et al.*, 1984b); FeLV-C/FA27, FeLV-C/FZ215, FeLV-C/FS246, FeLV-A/C-Sarma and FeLV-A/C-215 (Rigby, 1989; Rigby *et al.*, 1992).

Table 1.2 The diseases associated with FeLV infection

Malignant haemopoietic diseases	
Lymphomas	
alimentary	30% FeLV positive
multicentric	60% FeLV positive
thymic	80% FeLV postive
lymphatic leukaemia	60% FeLV positive
Leukaemia	
Many types including :- myeloid leukaemia erythroleukaemia reticuloendotheliosis	>90% FeLV positive
Non-malignant haemopoietic disease	
Anaemia	
Haemopoietic aplasias - erythroid or myeloid	
Immunosuppression	
Medullary osteosclerosis	
Myelofibrosis	
Others	
Glomerulonephritis	
Infertility	
Neurological Disorders	
General malaise, diarrhoea, weight loss etc.	

This last category includes a range of disorders found in FeLV infected cats but often the causal link has been only circumstantial.

(Derived from Hardy, 1980a; Jarrett, 1984; Jarrett, 1985)

been shown that FeLV TM is immunosuppressive *in vitro* affecting both granulocyte and lymphocyte lineages (Mathes *et al.,* 1978; Lafrado *et al.,* 1987). The relevance of these studies *in vivo* has not been determined.

The generation of proximal pathogens may also be responsible for immunodeficiency. A variant replication-defective form of FeLV-A, FeLV-61C, has been isolated which causes a fatal immunodeficiency disease in cats (Mullins *et al.,* 1986). This isolate is also cytopathic for the feline T cell line, 3201B (Overbaugh *et al.,* 1992). FeLV-61C has also been termed FeLV-FAIDS, which has caused a degree of confusion since the discovery of FIV and its associated disease complex. The helper virus isolate (FeLV-61E) that was obtained from the same cat, is also of the subgroup A phenotype.

No other groups have isolated viruses similar to FeLV-61C, which suggests that the generation of such viruses may be an uncommon event. Inoculation of 61E does not induce immunodeficiency suggesting that immunodeficiency variants do not commonly arise from this isolate *in vivo* (Overbaugh *et al.,* 1988a; Donahue *et al.,* 1991; Overbaugh *et al.,* 1992).

The mechanism by which FeLV-61C causes immunodeficiency and T cell cytopathicity has been extensively investigated. Molecular cloning and subsequent sequencing of the 61C isolate revealed differences in the *env* gene chiefly in the VR 1 and VR 5 regions. Using a large number of recombinants of 61C and its subgroup A helper virus (FeLV-61E), the regions of the envelope gene responsible for T cell killing and immunodeficiency have been identified (Donahue *et al.,* 1991). Replacement of the *gag* and *pol* genes of 61C with those of 61E produces a recombinant virus which retains the cytopathic properties of 61C, but is replication competent (FeLV-EECC). The nature of the immunodeficiency induced by the virus has been shown to be initially due to lymphocyte subset alterations, specifically in the CD4+ lineage (Quackenbush *et al.,* 1990). The mechanism by which these sequences exert their effects has been suggested to be a failure to develop superinfection interference, which results in the accumulation of unintegrated viral DNA within the cells (Mullins *et al.,* 1986; Reinhart *et al.,* 1993). This may lead to the accumulation of envelope proteins within the cell. It is known that differences in the envelope protein of 61C result in delayed processing and retention of the envelope proteins within the cell (Poss *et al.,* 1989; Poss *et al.,* 1990). The viral determinants of the failure to establish superinfection interference appear to localise to the same regions as those responsible for T cell killing and immunodeficiency (Reinhart *et al.,* 1993).

It has recently been shown that subtle differences in the ecotropic murine leukaemia

virus (E-MuLV) receptor protein, the principal mammalian basic amino acid transporter (Albritton *et al.,* 1989; Kim *et al.,* 1991; Wang *et al.,* 1991), can effectively block the entry of one strain of E-MuLV into a murine fibroblast cell line (Eiden *et al.,* 1993). This result suggests that retroviruses in the same receptor group interact in different ways with that receptor and therefore can exhibit differences in host range. This result would also suggest that the failure of FeLV-61E to establish superinfection interference against the replication competent FeLV-61E/61C chimera (FeLV-EECC) (Reinhart *et al.,* 1993) could be due to a failure of interference at the level of the VCR/VAS interaction, despite utilisation of a common receptor. This is in contrast to the postulated existence of a secondary, virus-specific, receptor (Reinhart *et al.,* 1993).

(b) FeLV induced oncogenesis

FeLV may cause neoplastic development by a variety of distinct mechanisms including transduction of the *myc* oncogene, integration of FeLV into the *myc* gene (Neil *et al.,* 1984) and the generation of oncogene-containing feline sarcoma viruses (FeSVs) (Snyder and Theilen, 1969). A comprehensive review of this field has been published recently (Rezanka *et al.,* 1992).

(c) FeLV induced pure red cell aplasia

Pure red cell aplasia (PRCA) is one of the most acute, degenerative retrovirus induced diseases known (Hoover *et al.,* 1974). Newborn kittens infected experimentally with biologically or molecularly cloned isolates of FeLV-C rapidly become anaemic and die (Onions *et al.,* 1982; Riedel *et al.,* 1986). However, if 8 week old kittens are infected experimentally with FeLV-C they do not become viraemic or develop disease. In contrast control animals of a similar age are susceptible to FeLV-A (Jarrett *et al.,* 1984). This age-related restriction can be by-passed by inoculation of the virus into the bone marrow (Dornsife *et al.,* 1989) or using FeLV-A as a helper virus (Jarrett *et al.,* 1984).

From experiments involving *in vitro* bone marrow cultivation, the anaemia has been demonstrated to be due to a failure in the maturation of erythroid progenitors (Testa *et al.,* 1983; Abkowitz *et al.,* 1987a). The myeloid compartment of the bone marrow is unaffected until the terminal stages of the disease. The exact role of FeLV-C in the development of PRCA is not clear. It is not due to the clonal expansion of a transformed myeloid cell (Abkowitz *et al.,* 1985) and lacks a defined immunological component (McAllister *et al.,* 1972). The early progenitors do become more sensitive to heterologous complement, but these changes are not seen with cat serum (Abkowitz *et al.,* 1987b). It has been demonstrated that FeLV-C, like FeLV-A, replicates in a wide range of haematopoietic cells (Dean *et al.,*

1992). These authors failed to demonstrate any significant difference in the number of cells infected by each subgroup. However, early erythroid progenitors are rare and so the analysis of whole bone marrow in this fashion may be insufficiently sensitive. It has been suggested that PRCA is a model for aplastic anaemia in humans (Sen *et al.,* 1978; Riedel *et al.,* 1986). However, although the pathological findings are similar, the latter condition is a pan-cell aplasia with a well defined immunological basis.

(2) Immunity to FeLV

Innate immunity plays an important role in the epidemiology of FeLV infections. A gradual decline in the susceptibility of cats to FeLV infection is seen with increasing age (Hoover *et al.,* 1976). If newborn kittens are experimentally infected with FeLV then they all become persistently viraemic. If the experiment is repeated with 8 week old cats then approximately 85% become persistently viraemic. Cats of 16 weeks of age are less susceptible to FeLV: about 15% become persistently viraemic when experimentally challenged (Hoover *et al.,* 1976). Others (Jarrett *et al.,* 1982) have found a similar age-related restriction, though the exact results differ from those given above. The marked age-related susceptibility to the development of persistent viraemia probably explains why most FeLV viraemic cats are under 6 years old (Hosie *et al.,* 1989). In experimental infections, immunosuppressive therapy with prednisolone at 10 mg/kg abrogates this age related resistance (Rojko *et al.,* 1979a).

Passive immunity, derived from maternal antibodies to FeLV, is known to exist (Hoover *et al.,* 1977; Jarrett *et al.,* 1977). Resistance to infection can also be experimentally induced by the transfer of hyperimmune neutralising goat anti-FeLV serum up to 6 days after challenge with the virus (deNoronha *et al.,* 1978). These results suggested that the presence of neutralising antibodies alone were sufficient to confer resistance to the development of persistent viraemia.

Virus neutralising antibodies have been demonstrated in the sera of recovered cats and have been presumed to be the major method by which cats resist subsequent challenges (Sarma *et al.,* 1974; Schaller and Olsen, 1975; Russell and Jarrett, 1978a). In the absence of experimental challenge, they are the best marker for the FeLV-immune cat. Detectable titres do not appear until 6 to 8 weeks post-infection. This finding suggests that other, earlier, mechanisms may also play a role in preventing the onset of viraemia. These mechanisms may include cytotoxic T cells (CTL). As assays for CTL activity in the cat are currently being developed this possibility has yet to be investigated fully. A review of the roles of T cells, natural killer cells, complement and interferon in the control of FeLV viraemia has been

published (Rojko and Olsen, 1984).

(3) The Prevention of FeLV Infection

Approximately 80% of cats that are persistently viraemic with FeLV die within 3 years of diagnosis (McClelland *et al.* 1980). In situations where close social contact exists between cats, such as closed multiple cat households, FeLV prevalence rates of up to 90% are found (Jarrett, 1984). These observations indicate a need for an effective method of controlling FeLV in the field. A test and removal scheme (Hardy *et al.,* 1976) has been used successfully in many breeding catteries (Weijer and Daams, 1978). The continuing success of the scheme is dependent upon the isolation of the cats from sources of infection. Where such conditions do not exist, vaccination represents the only available method for controlling FeLV.

(4) Vaccination against FeLV

FeLV vaccination should be considered a distinct possibility because many naturally exposed cats become immune (Russell and Jarrett, 1978a). Immunization with purified envelope glycoprotein is known to be sufficient to induce virus neutralising antibodies (Salerno *et al.,* 1978). By analogy with Friend murine leukaemia virus it is likely that only very small quantities of pure, non-denatured, envelope glycoprotein are required (Hunsmann *et al.,* 1975). It is also known that the presence of neutralising antibodies alone is sufficient to protect cats against the development of viraemia (Jarrett *et al.,* 1977; Hoover *et al.,* 1977; deNoronha *et al.,* 1978). The inference from these studies is that the ideal FeLV vaccine would consist of the purified envelope glycoprotein and would induce virus neutralising antibodies prior to challenge. A number of problems in obtaining this ideal have been identified and have ensured that no currently available vaccine is universally regarded as having achieved this ideal, though most do seem to provide some protection against the development of persistent viraemia (Haffer *et al.,* 1987; Marciani *et al.,* 1991; Hoover *et al.,* 1991; Hines *et al.,* 1991; Tizard and Bass, 1991; York and York, 1991).

Early studies did not attempt to purify the envelope glycoprotein but instead utilised low doses of live virus, whole killed virus or infected cells (Jarrett *et al.,* 1975; Pedersen *et al.,* 1979; Salerno *et al.,* 1979). The use of live virus is considered to be too dangerous for routine commercial use. However the experimental vaccines were effective (Jarrett *et al.,* 1975; Salerno *et al.,* 1979). Vaccines containing formaldehyde inactivated virus (Pedersen *et al.,* 1979) or heat inactivated virus (Olsen *et al.,* 1976) were not successful. This may reflect the denaturing effect of certain methods of fixation on the envelope glycoprotein. A subsequent formalin inactivated preparation was more successful (Pedersen *et al.,*

1986). There are now several inactivated virus vaccines commercially available in the USA (Hoover *et al.,* 1991; Tizard and Bass, 1991; Hines *et al.,* 1991; York and York, 1991). One of these vaccines is reported to induce pre-challenge virus neutralising antibodies (York and York, 1991). However this study was flawed by the use of non-SPF cats whose prior exposure to FeLV was not fully ascertained by testing for FOCMA antibodies. No whole inactivated FeLV vaccines can therefore be regarded as being completely protective. The potential immunosuppressive properties of vaccines containing the TM component of the FeLV *env* gene (Mathes *et al.,* 1978) have, to date, not been demonstrated by experimental data.

The use of FeLV-infected cells as FeLV vaccines has produced equivocal results; some workers have reported failure (Pedersen *et al.,* 1979), whilst others have described more encouraging results (Jarrett *et al.,* 1975). Protection against the development of FeSV-induced fibrosarcomas was observed following the vaccination of cats with FeLV-infected cells but not against the development of viraemia with FeLV (which was present as a helper virus) (Grant *et al.,* 1980).

The low yields of envelope glycoprotein initially precluded the use of this viral component as a commercial vaccine. However, the production of envelope glycoprotein from virus infected cells can be increased *in vitro* with the use of serum-free culture medium (Lewis *et al.,* 1981). This technique formed the basis of the first commercial FeLV vaccine (Leukocell; Norden). This vaccine was subjected to extensive trials (Mastro *et al.,* 1986; Sharpee *et al.,* 1986). Subsequently the vaccine was refined and is now marketed as Leukocell 2 (Haffer *et al.,* 1987). The efficacy of both vaccines has been questioned by other workers (Pedersen *et al.,* 1985; Kumar *et al.* 1989; Hoover *et al.,* 1991; Tizard and Bass, 1991; Sebring *et al.,* 1991). Furthermore the first commercial FeLV vaccine had a relatively high incidence of severe adverse reactions (Pollock and Haffer, 1991).

The next logical step for vaccine researchers was to increase the quantity of envelope protein by using recombinant DNA technology. The first recombinant FeLV envelope protein was produced using a bacterial expression system (Marciani *et al.,* 1991). The product was non-glycosylated and required high concentrations of urea for solubilisation. As recombinant soluble proteins are often poorly immunogenic, the production of recombinant envelope glycoprotein requires the use of highly effective adjuvants. The successful use of native gp70 incorporated into immune stimulating complexes (ISCOMs) has been reported (Osterhaus *et al.,* 1985; Osterhaus *et al.,* 1989). The vaccine induced virus neutralising antibodies in cats prior to challenge with FeLV. These studies are the only description of an FeLV vaccine which consistently induces prechallenge virus neutralising antibodies. However, significant losses of the envelope glycoprotein during the

purification process may preclude the use of this preparation as a commercial vaccine (Akerblom *et al.,* 1989; Osterhaus *et al.,* 1989). Increasing the efficiency of antigen incorporation into the ISCOMs by palmitification of the antigen (Reid, 1992), or a recombinant version thereof, may yet produce a reliable and cost-efficient vaccine.

The development of synthetic peptide vaccines corresponding to putative neutralising epitopes of the FeLV SU has not yielded much success. Vaccination of rabbits with these peptides produced virus neutralising antibodies, but these results could not be repeated in cats (Elder *et al.,* 1987; Nick *et al.,* 1990; Kumar *et al.* 1989; Weijer *et al.,* 1993). This may reflect a genetic restriction in cats to certain epitopes. It is known that minor changes in amino acid sequence outside of the antibody binding region affect the binding of neutralising monoclonal antibodies (Nicholaisen-Strouss *et al.,* 1987).

There have been several attempts to develop a live recombinant virus vaccine vector (Gilbert *et al.,* 1986; Wardley *et al.,* 1992; Tartaglia *et al.,* 1993). These vectors rely on infection of the cats own cells to make the FeLV envelope glycoprotein. This protein should therefore be identical to the protein that is produced during natural FeLV infection. The vector-derived protein may also be presented to the immune system in a similar fashion to native FeLV envelope protein in a natural challenge. Gilbert *et al.* (1986) generated a recombinant vaccinia virus which contained the *env* gene of FeLV-B. The protein was expressed *in vitro* but a lack of immunogenicity was observed following inoculation into both cats and mice. The reason for this lack of immunogenicity was not determined. Subsequently, a recombinant feline herpesvirus containing the FeLV *env* and *gag* genes was demonstrated to express a product *in vitro* (Cole *et al.,* 1990). However, on testing in cats, detectable anti-gp70 antibodies were only induced when a recombinant protein (produced in the baculovirus system) was included with the recombinant virus in the vaccination schedule (Wardley *et al.,* 1992).

Recently the use of a recombinant canarypox virus as a vaccine vector has been reported (Tartaglia *et al.,* 1993). Inoculation of this virus into five cats did not generate any virus neutralising antibodies. However, subsequent challenge of these cats with FeLV failed to establish persistent viraemia. In contrast, all the control cats became persistently viraemic. To prevent the establishment of persistent viraemia without the development of virus neutralising antibodies reinforces the concept of T cell effector mechanisms playing an early role in virus elimination. However vaccines need to be effective over long periods of time and, in the absence of virus neutralising antibody, it is not clear how this is to be assessed.

Cats are most susceptible to the development of persistent viraemia following a challenge with FeLV once maternal antibody has waned but before the development of age related resistance, that is between 6 and 16 weeks of age. As the age related resistance can be overcome experimentally using corticosteriods (Rojko *et al.,* 1979a), it is suggested that in high exposure situations the phenomenon may only provide some protection. The existence in the cat population of some FeLV positive individuals that are old (Hosie *et al.,* 1989) is probably a testimony to this, although long term carrier status cannot be discounted. These findings suggest that the most appropriate time to vaccinate a cat is early in life, but that vaccination may be considered at any age.

In summary, two major problems confront an aspiring FeLV vaccine manufacturer. Firstly, quantity of envelope glycoprotein, and secondly, the availability of an effective, non-toxic adjuvant.

1.3 Viral Cellular Receptors

1.3.1 General Introduction

Every virus, in order to replicate, must gain entry to a host cell. Advances in molecular biology techniques have permitted the detailed examination of the mechanisms by which viruses enter cells. Central to these studies has been the concept that a single molecule in the host cell membrane acts as an initial binding site for the virus. A variety of terms have been employed to describe these molecules and here the term 'viral cell surface receptor' (VCSR) shall be used. The adherence of one or several viral surface molecules (receptor binding sites : RBSs) to the VCSR is regarded as being a prerequisite for the entrance of the virus. The specificity of this reaction and the distribution of its participants represent major determinants in the pathogenesis of many viral diseases. It is highly improbable that VCSRs were evolved by the host cell for the benefit of the virus; rather they are normal components of the cell membrane and serve some useful function for the cell. The virus can be regarded as subverting these components for its own purposes. This section illustrates some of the concepts associated with viral cell surface receptors and describes the methods which have been employed to study them.

Following binding to a VCSR, the virus may enter by pH-dependent receptor-mediated endocytosis or pH-independent membrane fusion. Comprehensive reviews of these two processes and the involvement of the viral envelope proteins in them has been published (White *et al.,* 1983; Marsh and Helenius, 1989).

(1) Definition of a viral cell surface receptor

Viral cell surface receptors can be defined as single, specific cell surface molecules that are normal components of the cell membrane but which are directly used by a virus as an initial binding site. This definition does not include the units formed at a later stage of viral entry by the recruitment of other cell surface molecules; here these units are regarded as being part of the entry process and not part of the binding process. The term 'viral entry complexes (VECs)' could be employed to describe these later units. Not included in this definition either are 'virus receptors' which do not bind specifically to viruses, but rather to molecules such as antibodies which themselves have bound to viruses. For example, the infection of macrophages by several viruses is enhanced by the presence of anti-virus antibodies which bind to the virus and to the macrophage Fc receptor. The antibody-virus complex is then endocytosed. Viruses which are known to infect macrophages in this manner include dengue virus (Halstead and O'Rouke, 1977), feline infectious peritonitis virus (FIPV) (Olsen *et al.,* 1992) and HIV (Takeda *et al.,* 1988). It has been suggested that antibody-dependent enhancement of FeLV infection may occur (Nick *et al.,* 1990; Pedersen *et al.,* 1986). In these instances, these viruses do not possess a cell surface receptor. Equally, in some circumstances, viruses may be able to use several cell surface receptors on a given cell type.

Many viruses have had cell surface receptors assigned to them. Lentz (1990) lists 25 viruses with their corresponding VCSRs. Most of these VCSRs are only vaguely described, for example 'sialoglycoproteins' for encephalomyocarditis virus. Alternatively, several reports may give conflicting information, for example, Hepatitis B may use the polymeric IgA or serum albumin receptors on hepatocytes (Machida *et al.,* 1984; Neurath *et al.,* 1986). It is possible that both molecules could function as receptors. A few VCSRs are well defined and these are listed in Table 1.3.

The possession of a receptor confers on the cell nothing more than an ability to bind the virus. Mere interaction of the RBS and its corresponding cell surface receptor is insufficient to ensure active viral penetration or productive infection. Murine cells, if transfected with the cDNA encoding for CD4, develop the ability to bind HIV but will not allow the virus to replicate (Maddon *et al.,* 1986). These interactions are, however, necessary preconditions for both viral penetration and productive infection. Even if a cell possesses a receptor, the virus must still obtain access to the tissues. The importance of access to susceptible tissues was demonstrated a number of years ago when it was shown that vaccinia virus, which is normally taken up by the Kupffer cells of the mouse liver and does not infect the hepatocytes, can infect hepatocytes if it is injected into the mouse bile duct (Mims,

Table 1.3 Viral cell surface receptors

Virus	VCR	Reference
ENVELOPED		
HIV	CD4	Dalgleish *et al.*, 1984
Epstein-Barr virus	CR2 (C3d receptor)	Fingeroth *et al.*, 1984
Sindbis virus	High affinity laminin receptor	Wang *et al.*, 1992
Mouse hepatitis virus	Carcinoembryonic antigen	Williams *et al.*, 1990 Williams *et al.*, 1991
Transmissable gastro-enteritis virus	Aminopeptidase N	Delmas *et al.*, 1992
Human coronavirus 229E	Aminopeptidase N	Yeager *et al.*, 1992
Rabies virus	Acetylcholine receptor	Lentz *et al.*, 1982
Ecotropic murine leukaemia virus	Basic amino acid transporter	Albritton *et al.*, 1989 Kim *et al.*, 1991 Wang *et al.*, 1991
Vaccinia	Epidermal growth factor receptor	Eppstein *et al.*, 1985
Gibbon ape leukaemia virus	Phosphate transporter protein	O'Hara *et al.*, 1990 Johann *et al.*, 1992
NON-ENVELOPED		
Echovirus	Integrin VLA-2	Bergelson *et al.*, 1992
Poliovirus	Novel member of immunoglobulin superfamily	Mendelssohn *et al.*, 1989
Rhinovirus	ICAM-1	Greve *et al.*, 1989 Staunton *et al.*, 1989

1960). A VCSR is present, though not normally accessible.

(2) The biochemical nature of the VCSR

Individual VCSRs have been identified from all 3 major groups of cell structural components, namely proteins, lipids and carbohydrates (including glycosaminoglycans). Molecules containing sialic acid are used by a number of viruses such as influenza, polyoma and encephalomyocarditis viruses (Lentz, 1990).

Proteins which act as VCSRs have aroused the most interest in recent years. In a few cases viruses have enabled the identification of previously unidentified cell surface proteins, for example the poliovirus cell surface receptor is a novel member of the immunoglobulin superfamily (Mendelson *et al.,* 1989). In other cases viruses have been found to use previously characterised molecules. For example, the receptor for gibbon ape leukaemia virus (GALV) was found to be a phosphate permease that had previously been identified in *Neurospora crassa* (Johann *et al.,* 1992).

(3) Quantification of receptors

The choice of which method to use to attempt to identify a viral cell surface receptor will, to some extent, depend on the quantity of receptor available. The determination of the number of VCSRs per cell is hampered in many cases by the minimum requirement for viral entry being greater than one receptor. The difference may be due to the need to form viral entry complexes. HeLa cells, which are susceptible to poliovirus, have 1 x 10^5 binding sites per cell for the poliovirus VCSR-specific monoclonal antibody D171. However, as determined by viral assays (which rely on viral entry), HeLa cells have only 3000 poliovirus binding sites (Mendelson *et al.,* 1989). Experiments involving competition between isolated viral glycoprotein and virus for a VCSR, showed that there were approximately 5 x 10^5 MuLV gp71 binding sites per cell (DeLarco and Todaro, 1976). It has also been demonstrated that HIV requires multiple gp120 molecules (and by implication multiple CD4 molecules) for infection (Layne *et al.,* 1990).

1.3.2 The Importance of Studies on Viral Cell Surface Receptors

(1) Anti-viral treatment

Some viruses, particularly those which exhibit a large degree of antigenic diversity or a high rate of antigenic change, may never be readily controlled by vaccination; for example, HIV. These viruses may have to be controlled by anti-viral drugs. At present most antiviral agents act on the virus once it has penetrated the cell.

Examples of such drugs include AZT and acyclovir. An ideal antiviral agent would prevent viral entry into the cell. Such agents would presumably act either at the level of the VCSR/RBS interaction or the subsequent viral penetration. To date, although *in vitro* results have been encouraging, *in vivo* results have been disappointing. A detailed description of agents which might be able to interfere with the VCSR/RBS interaction can be found in a recent review (Lentz, 1990).

(2) Viral pathogenesis

The RBS/VCSR interaction of some viruses may help to understand the pathogenesis of their diseases. For example, the use of the acetyl-choline receptor of synaptic membranes by rabies virus may explain the neurotropism exhibited by the virus (Lentz *et al.,* 1982). Similarly the functional consequences of vaccinia virus entering the host cell by means of the epidermal growth factor receptor are amply demonstrated by the resulting clinical disease (Eppstein *et al.,* 1985). The underlying mechanisms of other viral diseases are less amenable to explanation in terms of the RBS/VCSR interaction: for instance the employment of the widespread intercellular adhesion molecule 1 (ICAM-1) by the human rhinovirus, the causative agent of the common cold, does not readily explain the nature of the disease produced by the virus (Greve *et al.,* 1989; Staunton *et al.,* 1989).

(3) Autoimmunity.

It has commonly been observed that viruses bind to molecules at sites which are themselves sites for natural body ligands. These observations suggest that there may be similarities between the RBS and host cell proteins. Some similarities have been identified, such as those between Coxsackie B4 virus and myocardial cells, with implications in the pathogenesis of autoimmune disease (Oldstone, 1987). By similar reasoning it is possible to envisage that this similarity may benefit the virus by avoiding the direction of an immune reaction to those portions of its external proteins that are involved in host cell binding (Oldstone, 1987).

1.3.3 Methods of Identifying Receptors

(1) Blockage of viral infection with other ligands

Conceptually the simplest method to identify a VCSR is to use a ligand with a known receptor specificity to block virus infection.

Such an approach, however, is fraught with the potential for misinterpretation. Ligands can exert their anti-virus binding effects by a variety of routes other than simple blockage. The value of α-bungarotoxin in identifying the rabies virus receptor (Lentz *et al.,* 1982) was questioned for several years until conclusive proof

was later provided (Lentz *et al.,* 1987).

Isolated FeLV-A SU glycoprotein has been used as a ligand for the putative VCSR for FeLV-A (Ghosh *et al.,* 1992). This study is discussed further in Chapter 6.

(2) Blockage of viral infection with monoclonal antibodies

A common way of identifying VCSRs is to block viral infection with an anti-cell monoclonal antibody. The process is greatly simplified if the identity of the cell surface ligand recognised by the monoclonal antibody is already known.

The identification of the C3d complement receptor (CR2), a membrane-spanning glycoprotein, on the surface of B lymphocytes as the Epstein-Barr virus VCSR was the first receptor to be identified by this method (Fingeroth *et al.,* 1984). The monoclonal antibody used was of known specificity and the cell tropism of the virus had suggested that CR2 might well serve as the VCSR. Similarly, the known T helper cell tropism of HIV greatly facilitated the identification of CD4 as its major receptor (Dalgleish *et al.,* 1984).

As an alternative to this strategy, monoclonal antibodies can be prepared specifically for their ability to block viral infection by injection of susceptible cells into mice. Having identified such a monoclonal antibody it may be possible to identify its ligand directly; for example, integrin VLA-2 was identified as the receptor for echovirus 1 in this fashion (Bergelson *et al.,* 1992). Alternatively, the ligand can be purified by affinity chromatography and then amino acid sequencing can be used to identify a homologue. The identification of ICAM-1 as the major human rhinovirus receptor by one group relied on this technique (Greve *et al.,* 1989), while another group used a monoclonal antibody of known specificity (Staunton *et al.,* 1989). ICAM-1 is a member of the immunoglobulin superfamily that interacts with the LFA-1 receptor on T cells. An essentially similar technique was used to identify aminopeptidase-N as the receptor for two coronaviruses in the same serogroup, transmissible gastroenteritis virus (TGEV) (Delmas *et al.,* 1992) and human coronavirus 229E (HuCo229E) (Yeager *et al.,* 1992). The receptor for mouse hepatitis virus (MHV), a third, unrelated, coronavirus, has been identified as a member of the carcinoembryonic antigen family (Williams *et al.,* 1990; Williams *et al.,* 1991).

A disadvantage of this technique is that monoclonal antibodies which block virus adsorption do not always bind to the cell surface receptor (Thiry *et al.,* 1983; Sommerfelt *et al.,* 1990). A further disadvantage is that a large number of hybridomas may have to be screened for the production of monoclonal antibodies that not only bind to the receptor, but also block infection. For example, 700

hybridomas were screened for their ability to produce antibodies that blocked rhinovirus infection (Greve *et al.,* 1989).

(3) Anti-idiotype antibodies

It is often easier to determine the identity of the RBS, than the identity of the VCSR. By generating antibodies to the RBS and subsequently generating anti-idiotype antibodies it might be possible to identify the VCSR. This approach has been attempted for Sindbis virus (Wang *et al.,* 1991). The anti-idiotypic antibody identified a 63 kDa protein which was later demonstrated to be a minor, species-specific, Sindbis virus receptor. The major Sindbis virus receptor was identified, using an anti-susceptible cell monoclonal antibody, as the high affinity laminin receptor (Wang *et al.,* 1992).

A recent study of the recognition of CD4 by antibodies and HIV gp120 questioned the usefulness of antibodies as mimics for RBSs (Davis *et al.,* 1992). These authors suggested that there were no reports of anti-idiotype antibodies unambiguously identifying a VCSR. The results obtained with the Sindbis virus anti-idiotype antibodies are therefore likely to be the exception rather than the rule. Polyclonal anti-idiotype antibodies have been prepared using FeLV gp70 which partially block the infection of susceptible cells (Tavares *et al.,* 1991).

(4) Transfection of cDNA

In recent years several VCSRs have been definitively identified by the transfection of susceptible cell cDNA into insusceptible cells. These VCSRs include the receptors for poliovirus (Mendelson *et al.,* 1989), ecotropic murine leukaemia virus (Albritton *et al.,* 1989) and gibbon ape leukaemia virus (O'Hara *et al.,* 1990). It is interesting to note that two retroviral cell surface receptors have been identified in this fashion.

To identify a receptor using this method, it is first necessary to demonstrate that transfection of viral DNA or RNA into an non-susceptible cell is sufficient to establish an infection and therefore the VCSR represents the only barrier to infection of the cell. Secondly, it is important to have a means of identifying the receptor positive cells. Anti-cell monoclonal antibodies that block viral infection, but which, for whatever reason, cannot be used to isolate the receptor, are effective markers (Mendelson *et al.,* 1989). Retroviral vectors carrying a drug resistance gene can be used in the identification of retroviral receptors (Albritton *et al.,* 1989; O'Hara *et al.,* 1990).

Transfection of cDNA avoids the difficulties caused by the lability of receptor

(Mendelson *et al.,* 1989) but requires the screening of large eukaryotic and prokaryotic cell libraries. The cloning of the receptor gene is, however, a distinct advantage. Such a clone can be used to examine other host tissues for the expression of receptor transcripts by using northern hybridization analyses (Mendelson *et al.,* 1989). Another advantage of this technique is that no knowledge is required of the functional nature of the receptor. The technique is not applicable to those viruses which have specific, post-entry, requirements for productive infection.

1.3.4 Retrovirus Cell Receptors

(1) General Comments

The receptors for several retroviruses have now been conclusively identified. The best characterised of these is the interaction of HIV SU with CD4, (McDougal *et al.,* 1991) which is discussed below. Using recombinant retroviral vectors the cell surface receptors for gibbon ape leukaemia virus (GALV) and ecotropic murine leukaemia virus have been identified as being a phosphate permease protein and a basic amino acid transporter respectively (Albritton *et al.,* 1989; O'Hara *et al.,* 1990; Kim *et al.,* 1991; Wang *et al.,* 1991; Johann *et al.,* 1992). Preliminary studies have also been performed on a number of other putative retrovirus receptors (Hosie *et al.,* 1993; Ghosh *et al.,* 1992).

Early in the study of retroviruses it was noted that cell lines infected with one retrovirus were not susceptible to other isolates of the same retrovirus (Rubin, 1960). This phenomenon, referred to as superinfection interference, is not unique to retroviruses but has been extensively used in this field. Since some retroviruses can infect cell lines of non-host origin *in vitro*, superinfection interference can be investigated between retroviruses from different species. Retroviruses can therefore be grouped according to their receptor usage, without at the same time knowing the nature of the receptor. At least 8 receptor groups exist on human cells for retroviruses (Sommerfelt and Weiss, 1989). Using the information that FeLV-B and GALV interfered with each other on human cells, it was possible to identify the receptor on human cells for FeLV-B as the same phosphate permease protein that used by GALV (Takeuchi *et al.,* 1993). It remains to be confirmed whether the receptor for FeLV-B on feline cells is a homologue of this human protein.

Recently, studies on the identification of the FIV cell surface receptor have been described (Hosie *et al.,* 1993, Willett *et al.*, in press). These studies used an anti-cell monoclonal antibody that blocked FIV infection. It has yet to be demonstrated that the monoclonal antibody blocks infection at the level of the VCSR. The cell surface component recognised by the monoclonal antibody is not thought to be the

feline homologue of CD4 (Brown *et al.,* 1991; Hosie *et al.,* 1993) but rather the feline homologue of CD9 (Willett *et al.*, in press).

The existence of endogenous retroviruses, such as RD-114, which are unable to infect their own host cells but can, experimentally, infect non-host cell lines may represent an historical record involving incarceration following VCSR mutation.

(2) HIV / CD4

Several methods have been used to demonstrate that HIV uses CD4 as its major receptor. The original papers (Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984) reported the testing of the susceptibility of different cell types to pseudotypes of vesicular stomatitis virus bearing the retroviral envelope antigen. It was also demonstrated that the common determinant for rosette formation between virus infected and susceptible cells was the expression of the CD4 molecule. Furthermore, 155 different monoclonal antibodies were tested for their ability to block both the rosette and the pseudotype assay. Each of 14 anti-CD4 antibodies blocked both rosette and pseudotype formation. Preincubation of CD4+ cells with anti-CD4 monoclonal antibodies also blocked infection by HIV (Klatzmann *et al.,* 1984).

The ability of HIV to infect CD4+ cells explains the T cell tropism of the virus. It also explains the infection by HIV of a wide range of cells that also express CD4 (Weiss, 1991). The CD4 antigen is a member of the immunoglobulin superfamily (Maddon *et al.,* 1986) and has a variety of functions. It is involved in immune recognition and stabilises the interaction of the T cell receptor with MHC class II molecules (Janeway, 1988). The binding of gp120 to CD4 blocks many of its immune reactions and the subsequent viral replication is believed to be responsible for many of the immunological abnormalities noted in HIV infected individuals (Rosenberg and Fauci, 1991). However, certain aspects of the pathogenesis of HIV infection are not so readily explained in terms of HIV / CD4 interaction (Weiss, 1991). There is also evidence that CD4 is not the receptor for HIV in the brain (Clapham *et al.,* 1989). It is known that HIV-2 and SIV also employ CD4 as a cell surface receptor (Sattentau *et al.,* 1988).

The interaction of CD4 and the envelope surface protein of HIV (gp120) has been analysed in great detail. The site of CD4-binding activity on gp120 has been identified as amino acid residues 422-437 and the site of gp120 binding on CD4 has been localised to the V1 domain (Habeshaw *et al.,* 1990; McDougal *et al.,* 1991). The gp120-binding site on CD4 is close to the MHC class II binding site and a region of gp120 near to the CD4 binding site is partially homologous to MHC class II. These observations together suggest that the viral attachment site (RBS) of HIV

may mimic the natural ligand for the VCSR. Furthermore, they suggest that the RBS may be not be antigenic in humans (Habeshaw *et al.*, 1990).

After HIV has bound to CD4 the mechanism by which the virus enters the cell is not known. A model has been proposed that involves conformational changes by both gp120 and CD4, as well as the transmembrane gp41 protein (Eiden and Lifson, 1992). It is known that HIV infection does not require the endocytosis of CD4 (Maddon *et al.*, 1988).

1.4 The Baculovirus Expression System

1.4.1 Baculoviruses: an Introduction

The family *Baculoviridae* contains several viruses of scientific and economic importance. They are the only group of viruses whose host range is restricted to the phylum *Arthropoda*, most members infecting the subphylum *Insecta*, though some have been isolated from *Crustacea*. The best studied and most commonly used baculovirus is the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) (Luckow and Summers, 1988). It is the type member of the family *Baculoviridae* and was originally isolated from a larva of the alfalfa looper (*Autographa californica*). Experimentally AcMNPV infects over 30 species of Leptopteran insects (moths, butterflies and their allies), chiefly when they are in the larval stage (Luckow and Summers, 1988).

AcMNPV is a rod shaped, enveloped virus approximately 350 nm in length (Federici, 1986). The 128 kb double stranded DNA genome is circular and is encased in a nucleocapsid (Luckow and Summers, 1988). Each virion may contain several nucleocapsids inside a single phospholipid based envelope. In the wild state several virions are encased in a protein capsule, called a polyhedron, which protects the virion against adverse environmental conditions external to its host. This capsule is made of a single protein called polyhedrin (Federici, 1986).

The life cycle of AcMNPV is complex, involving two forms of transmission, one intra-host and the other inter-host (Grandos and Williams, 1986). Following ingestion of the polyhedrin-encased virus, viral particles are released by a host enteric protease into the alkaline environment of the insect gut. After the virus has infected the epithelial cells of the insect's midgut, its genome is expressed in 4 stages (immediate-early, delayed-early, late and very late stages) (Cochran *et al.* 1986). The products of the last stage are 2 proteins: one, polyhedrin, with a relative molecular mass of 29,000 Da and another, p10, of 10,000 Da. The function of p10 is unknown, but it may be involved in cell lysis.

The infected cells release two forms of the virus; non-occluded and occluded. The non-occluded forms are released into the body fluids and can infect most of the insects cells. This allows the virus to spread rapidly throughout the body. The polyhedrin occluded virus cannot infect the host cells and plays little or no part in intra-host transmission.

The infection is usually fatal and once the insect is dead the polyhedrin-encased virions are released into the environment where they may persist for some time. In contrast the non-occluded virions are quickly inactivated in the external environment. Therefore the inter-host form of transmission, in contrast to the intra-host form, does require the virus to be encased in polyhedrin.

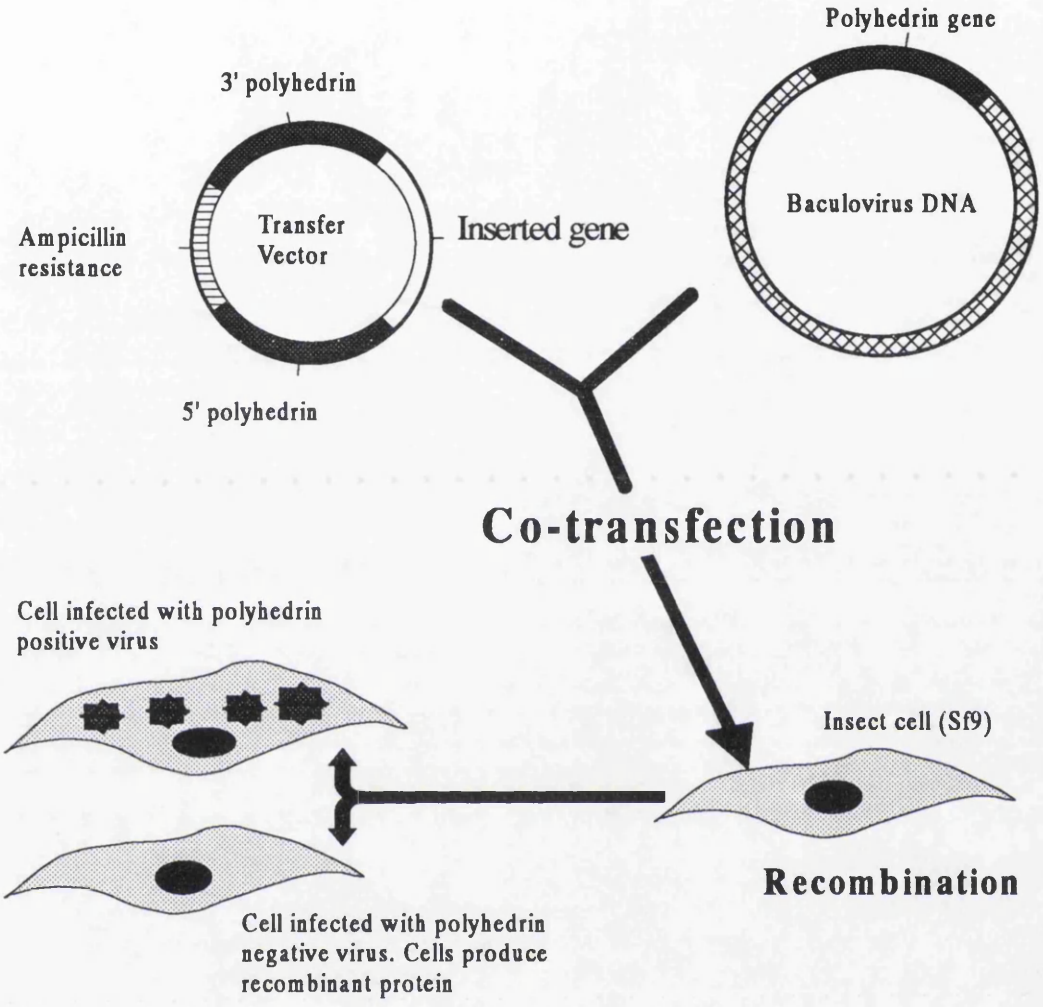
1.4.2 The Application of AcMNPV in the Baculovirus Expression System

Neither of the two late proteins, p10 and polyhedrin, are required for intra-host or *in vitro* transmission. The baculovirus expression system is based upon the replacement of these two non-essential genes with genes encoding for foreign proteins (Smith *et al.,* 1983a; Smith *et al.,* 1983b; King and Possee, 1992).

This is achieved by co-transfection of susceptible cells with wild type AcMNPV DNA and a recombinant plasmid which contains the foreign gene, flanked by extended polyhedrin gene sequences (Luckow and Summers, 1988). Homologous recombination occurs and the cells produce viral particles containing the foreign gene (see Figure 1.3). The only difficulty inherent in this system is distinguishing cells infected with the recombinant virus before all the cells are overcome with the occlusion positive, wild type virus. Several strategies have been developed for facilitating the purification of the recombinant, polyhedrin negative, stock away from its wild type counterpart. The use of *lacZ* as a reporter gene has been documented (Zuidema *et al.,* 1990; Pennock *et al.,* 1984). This system allows the cells infected by recombinant viruses to be visualised by staining with a chromogenic substrate.

The baculovirus expression system has two inherent safety features. As their host range is restricted to the phylum *Arthropoda*, the viruses are not pathogenic to vertebrates. The polyhedrin promoter has been demonstrated to be inactive in mammalian cells (Carbonell *et al.,* 1985). The system also avoids the use of transformed cells or transforming elements. Insect cells are simple to culture, requiring easily obtained media, no CO_2 enrichment, and relatively crude temperature control systems. The system can, with the aid of air-lift fermentation vats, easily be scaled up for industrial purposes (Luckow and Summers, 1988). The foreign protein is glycosylated by the insect cell and is often chemically and functionally similar to the wild type protein.

Figure 1.3 Schematic representation of the baculovirus expression system



The figure shows a schematic representation of the baculovirus expression system. A transfer vector containing the foreign gene of interest, flanked by sequences immediatly up- and downstream of the polyhedrin gene, is co-transfected with wild type baculovirus DNA into insect cells. Recombination occurs and a mixture of recombinant and wild type viruses are produced which can be separated by plaque purification.

An ampicillin resistance marker gene is included in the transfer vector to aid prokaryotic manipulation. Additional modifications to the system include a marker gene to aid selection of the recombinant viruses.

Many proteins have been produced in the baculovirus expression system. The more important examples are summarised in Table 1.4. For further information, Luckow and Summers (1988) list some 37 proteins that have been produced using the baculovirus expression system. Early vectors introduced the foreign gene downstream from the polyhedrin start codon resulting in the production of a fusion protein. Subsequently, altering the polyhedrin start codon from ATG to ATT allowed the high level production of non-fused proteins (Luckow and Summers, 1989).

The use of an alternative baculovirus expression system based upon the *Bombyx mori* nuclear polyhedrosis virus has also been described (Maeda, 1989). This system, which was originally developed for *in vivo* expression using silkworm larvae (Maeda *et al.,* 1985), may produce larger yields of certain proteins. The use of *Heliothus virescens* larvae and AcMNPV for *in vivo* expression is also documented (Kuroda *et al.*, 1989). The main advantage of such systems is the total quantity of protein that can be produced. The systems are also useful for studying the effects of certain foreign proteins on insect mortality.

1.4.3 Baculoviruses as Biological Control Agents

Baculoviruses are highly pathogenic to a number of commercially important insect pests. This has prompted attempts to use them to control insect numbers. Unlike many conventional pesticides they will not persist in the environment and cannot harm vertebrates. They do, however, suffer from certain drawbacks. They are expensive to produce and much of the virus is inactivated before it can infect the host. They are also insufficiently pathogenic allowing the insects to survive for up to 1 week after infection

In an attempt to overcome this latter obstacle workers at Oxford generated a recombinant baculovirus which expressed a scorpion toxin gene instead of its polyhedrin gene (Stewart *et al.,* 1991). This modification decreased the survival time of the infected individuals at the same time decreasing the LD_{50} values. The particular scorpion toxin chosen has no effect on mammals such as mice.

1.5 Project Aims

The identification of the FeLV cell surface receptor(s) will greatly assist the understanding of FeLV pathogenesis. In particular, the characterization of the FeLV-C cell surface receptor may help to explain the specific development of pure red cell aplasia induced by isolates of this subgroup.

The development of an effective FeLV vaccine will aid the control of this common,

Table 1.4 Some proteins produced in the baculovirus expression system

Viral proteins		**Reference**
HIV	*env*	Rusche *et al.*, 1987 (and others)
FeLV	*env*	Thomsen *et al.*, 1992
African horse sickness virus	VP7	Chuma *et al.*, 1992
Avian leukaemia virus	*env*	Noteborn *et al.*, 1990
FIV	*gag*	Morikawa *et al.*, 1991
Hepatitis B	Core antigen	Hilditch *et al.*, 1990
Influenza	Haemagglutinin	Kuroda *et al.*, 1986
Bacterial proteins		
E. coli β galactosidase		Pennock *et al.*, 1984
E. coli Chloramphenicol acetyl transferase		Carbonell *et al.*, 1985
Human proteins		
Interleukin-2		Smith *et al.*, 1985
β interferon		Smith *et al.*, 1983
c-myc		Miyamoto *et al.*, 1985

Adapted from Luckow and Summers (1988)

serious feline pathogen. When the work for this thesis began there were no commercial FeLV vaccines available in the UK. Although subsequently two vaccines were released, neither provides complete protection against the development of persistent viraemia.

The initial aim of this project was to produce recombinant FeLV SU from all three subgroups of FeLV and two FeLV-A/C chimeras in a protein expression system. Subsequently, the proteins were to be analysed using a variety of techniques to assess their functional characteristics in comparison to the native proteins.

The proteins were then to be used in an attempt to identify the cell surface receptor(s) for FeLV. Initially, the ability of the proteins to block FeLV infection was to be assessed and then co-precipitation studies with surface labelled cell lysates performed.

A subsidiary aim was to use the recombinant FeLV-A SU in vaccine studies.

Chapter 2. Materials and Methods

2.1 Materials

Details of reagents and methods commonly used are given below. Specific materials and methods are described in the relevant chapters.

2.1.1 Equipment

Plasticware was obtained from Costar or Nunc. All centrifuges and rotors used were made by Beckman. All other equipment manufacturers are named in the text.

2.1.2 Chemicals

All chemicals used were of Analar quality and, except where indicated, were obtained from BDH, NBL, Gibco BRL, Pharmacia or Sigma.

2.1.3 Standard Solutions

Acrylamide (30%) : Purchased from NBL as a solution containing 29 : 1 (w/w) acrylamide : methylene-bisacrylamide.

Agarose gel loading buffer : 10% Ficoll, 0.02% bromophenol blue, 0.02% xylene cyanol FF, 0.02% Orange G in water. To a given volume of sample was added a quarter volume of gel loading buffer.

Alkaline phosphatase (AP) buffer: 200 mM NaCl, 10 mM $MgCl_2$, 100 mM Tris (pH 9.5). Sterilised by autoclaving.

Ammonium persulphate : 10% (w/v) solution in water. Aliquots were stored at -20°C

Ampicillin : 100 mg/ml filter sterilised and stored in aliquots at -20°C. Used as a 1,000 x stock.

Blocking solution : TBS + 3% (w/v) Marvel (Chivers and Sons Ltd., Dublin).

Bromo-chloro-indolylphosphate (BCIP): 0.5 g dissolved in 10 ml of 100% DMF.

Coupling buffer : 10 mM $NaHCO_3$, 1 mM EGTA, pH 9.6. Filter sterilised.

Ethidium bromide : 10 mg/ml water. Stored in a light proof container.

2 x HEPES buffered saline : 280 mM NaCl, 50 mM HEPES, 3 mM Na_2HPO_4 (pH 7.05). Filter sterilised. The pH was checked periodically.

Klenow buffer : 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, filter sterilised and stored in

aliquots at 4°C.

L Agar : 15 g Bacto-agar added immediately before autoclaving 1 litre of L Broth (see below).

L Broth : 1% (w/v) tryptone, 0.5% (w/v) yeast extract in 90 mM NaCl. The suspension was sterilised by autoclaving prior to use and then glucose was added to a final concentration of 20 mM and $MgCl_2$ to a final concentration of 10 mM.

Lysozyme : 10 mg/ml in water. Made fresh when required.

Nitrobluetetrazolium (NBT) : 0.5 g dissolved in 10 ml of 70% DMF.

Phosphate buffered saline (PBS) : 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.2). Sterilised by autoclaving.

PBS-Tween : PBS + 0.05% (v/v) Tween 20.

PBS-EDTA : PBS + 0.5 mM EDTA (pH 7.2) with 0.005% phenol red as an indicator. Sterilised by autoclaving.

Protein gel loading buffer : 10% glycerol, 62.5 mM Tris (8.0), 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue in water. This was used as a 4 x stock.

SDS-PAGE stacking gel buffer : 125 mM Tris, 17 mM SDS (pH 6.8).

SDS-PAGE separating gel buffer : 375 mM Tris, 17 mM SDS (pH 8.8).

SDS-PAGE running buffer : 120 mM Tris, 0.5 M Glycine, 17 mM SDS.

STET solution : 1% Triton X100 (v/v), 20% sucrose (w/v), 5% EDTA (w/v) in 50 mM Tris (pH 8.0).

Streptomycin : 25 mg/ml filter sterilised and stored in aliquots at -20°C. Used as a 1,000 x stock.

TBE : 100 mM Tris, 100 mM orthoboric acid, 2.5 mM EDTA.

TE : 10 mM Tris, 1 mM EDTA.

Tris buffered saline (TBS) : 140 mM NaCl, 2.7 mM KCl, 25 mM Tris (pH 8.0). Sterilised by autoclaving.

TBS-Tween : TBS + 0.05% (v/v) Tween 20.

Western buffer : 25 mM Tris, 200 mM glycine in a 20% methanol/water solution.

X-Gal : 5-bromo 4-chloro 3-indolyl β - D galactoside was obtained from NBL.

Dissolved in DMF (80 mg/ml) and stored at -20°C.

2.1.4 Plasmids

(1) pFGA, pFGB, pFSC, pVC, pV215

The prototype proviral clones of FeLV-A, FeLV-B and FeLV-C, called pFGA, pFGB and pFSC respectively (Elder and Mullins, 1983; Stewart *et al.*, 1986; Riedel *et al.*, 1986), were obtained from Dr. M. Rigby (Department of Veterinary Pathology, University of Glasgow). pVC and pV215 are two proviral clones of chimeric A/C FeLVs created by site directed mutagenesis (Rigby, 1989). They both have an FeLV-A backbone but contain a small region near the 5' end of C-specific sequence which confers on their resulting, replication competent, viruses many of the properties of FeLV-C (Rigby *et al.*, 1992).

(2) pAcLacZ+

The plasmid pAcLacZ+ was obtained from Dr. R. Possee (Institute of Virology, Oxford) and is based on the plasmid pAcUW2(B) (Weyer *et al.*, 1990). It is used as a transfer vector for the baculovirus expression system and contains the polyhedrin promoter and flanking sequences. It also includes the *lacZ* gene under the control of the p10 promoter as a selectable marker. The backbone is a pUC8 derivative called pUC8/6/8 (Weyer *et al.*, 1990). Unlike the plasmid pAcUW2(B), it does not contain the actual polyhedrin gene.

(3) pBluescript SK+

This commercially available plasmid (Stratagene), which usually replicates to a high copy number, contains the α peptide portion of the *lacZ* gene under the control of the T3 promoter. The promoter and the α peptide segment are separated by a multiple cloning site which, if an insert is not present, allows α complementation when transfected into the DS941 host cells. If an insert is present then α complementation does not occur. This allows blue / white selection on agar plates impregnated with X-Gal. The plasmid also contains an ampicillin resistance gene.

(4) p1-10

This plasmid was obtained from Dr. M. Rigby. It contains the FIV/UK8 *env* gene in a pUC derivative. The 5' terminal 15 base pairs were derived from the Petaluma isolate. This produces one amino acid change (a glutamine instead of a glycine) from the predicted UK8 sequence. This amino acid change is in a conserved region of the protein (Rigby *et al.*, 1993).

2.1.5 Sera and Antibodies

(1) Rabbit serum #709

This serum was produced by Dr. G. Reid (Department of Veterinary Pathology, University of Glasgow) by immunization of a rabbit with FeLV glycoproteins. These proteins were prepared by lentil lectin affinity chromatography from detergent disrupted FeLV derived from FL74 culture fluid. The serum reacts well with native gp70 in Western blots and can also be used in ELISAs both as a capturing antibody or as a primary detection antibody.

(2) Rabbit serum #395

This is a serum derived from a specific pathogen free rabbit.

(3) Monoclonal antibodies

(a) 3-17 / 3-17AP / 6-15

These are anti-FeLV gp70 monoclonal antibodies, kindly donated by Dr. K. Weijer (Netherlands Cancer Institute). They all recognise the region covering amino acid residues 204 to 208 of FeLV-A (N.Spibey, personal communication), which has been shown to be an important neutralising epitope of gp70 (Elder *et al.,* 1987). They are neutralising *in vitro*; their titres are given in Table 2.1. They are of the IgG1 subclass (Weijer *et al.,* 1986). The 3-17AP MAb has alkaline phosphatase covalently linked to its Fc portion and was available as a purified antibody. The monoclonal antibodies 3-17 and 6-15 were available in ascites form.

(b) C1G10, HL2 and HL5

These are anti-FeLV gp70 monoclonal antibodies that were obtained from Dr.C.Grant (C1G10) and Professor H.Lutz (HL2 and HL5). C1G10 is reported to be FeLV/C-Sarma / FeLV/B-ST specific (Grant *et al.,* 1983). It does not neutralise several other FeLV/C isolates. It was available in a purified form. HL2 and HL5 are reported to be specific for FeLV-A/Glasgow-1 and a range of FeLV/C isolates (O.Jarrett, personal communication). They were available in ascites form.

The neutralising titres of these monoclonal antibodies *in vitro* are given in Table 2.1.

(c) VPG 68 and VPG 71.2

These monoclonal antibodies were prepared by Mr T. Dunsford (Department of Veterinary Pathology, University of Glasgow) against whole FIV virus prepared from FL4 cells. They both immunoprecipitate native FIV SU, but do not recognise

Table 2.1 Neutralisation titres of monoclonal antibodies to FeLV subgroups

a) FeLV-A, FeLV-B and FeLV-C

Subgroup and Strain	FeLV/A-Glasgow-1	FeLV/B-ST	FeLV/C-Sarma
C1G10	0	1280	1280
HL2	5120	4	800
HL5	320	20	200

b) FeLV-C (three isolates)

	FeLV/C		
	Sarma	FA27C	FS246
C1G10	3000	0	0
HL2	10000	20000	5000
HL5	40	5000	1280

The values presented in these tables were derived from separate experiments with different preparations of the antibodies.

the protein in western blots or ELISAs. VPG 71.2 also immunoprecipitates recombinant FIV / UK8 SU produced in the baculovirus system (A.Mackay, personal communication).

(4) Cat P11 / P12

These sera are derived from cats which recovered from natural contact infection with FeLV-A/Glasgow 1 (Madewell and Jarrett, 1983). The cats were kept in a barrier maintained cattery at the Department of Veterinary Pathology, University of Glasgow.

Both sera have neutralising antibodies *in vitro* with titres of 128 and 256 respectively. They react well with native gp70 on western blots. The serum P12 reacts specifically with FeLV-A gp70 in ELISAs. The subgroup specificity of serum P11 in ELISAs is unknown.

(5) Cat 31

This was a cat serum derived from the experimental infection of an 8 week old kitten with biologically cloned FeLV-C/Sarma. The kitten developed neutralising anti-FeLV-C/Sarma specific antibodies. The kitten was then boosted 8 months later with concentrated, purified FeLV-C/Sarma. The serum has high titres of anti-FeLV-C/ Sarma specific antibodies on testing with an ELISA, but low anti FeLV-A titres.

The neutralising antibody titres of this serum were 16 against FeLV-A/Glasgow-1, 16 against FeLV-B/ST and 1024 against FeLV-C/Sarma.

(6) Cat Wendy / Q25 / Ruth 2

These are specific pathogen free cat sera, obtained from animals kept in a barrier maintained cattery at the Department of Veterinary Pathology, University of Glasgow.

(7) Pooled FIV-infected cat serum P2

This serum was obtained by pooling the sera derived from a number of cats infected with UK8 isolate of FIV (Rigby *et al.,* 1993). The cats were kept in a barrier maintained cattery at University of Glasgow. The serum reacts well with FIV proteins on western blot.

(8) Goat serum

Goat serum that was used as a blocking antigen in western blots and ELISAs was obtained from Scottish Antibody Production Service. It was stored in 10 ml aliquots

at -20°C until required.

2.1.6 Viruses

(1) Baculoviruses

The baculovirus used, *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) (strain C6), was obtained from Dr. N. Stow (Institute of Virology, Glasgow).

(2) Feline Leukaemia Viruses

The FeLV strains used were; FeLV-A/Glasgow 1 (Jarrett *et al.,* 1973); FeLV-B/Snyder-Theilen (ST) (Sarma and Log, 1973); FeLV-C/FA27C (Onions *et al.,* 1982). They were obtained in either biologically or molecularly cloned form from Mr. M. Golder (Feline Virus Unit, University of Glasgow).

2.1.7 Bacterial Strains

(1) DS941

This is a strain of *E.coli* which has a partially deleted chromosomal *lacZ* gene and can be used for blue/white selection of recombinant plasmids containing the appropriate segment of *lacZ*. It is also resistant to streptomycin.

DS941 (Flinn *et al.,* 1989) is a derivative of AB1157 (Bachmann, 1972) and was obtained from Institute of Genetics, University of Glasgow. The genotype is *recF* $lacI^q$ *lacZΔ1M15*.

2.1.8 Eukaryotic Cell Lines

All cells were grown in media supplemented with 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. All the media used were supplied by Gibco BRL and kept at 4°C. All mammalian cells were cultured at 37°C and in air enriched with 5% CO_2. When necessary, a humified incubator was used. All adherent mammalian cells were subcultured by first washing with PBS-EDTA and then with 0.025% trypsin / PBS-EDTA mixture. After a short incubation at 37°C the cells were resuspended in fresh medium and replated. Cell were stored in a 10% DMSO / 90% FCS mixture in liquid nitrogen.

(1) AH927

This feline fibroblast cell line was derived from the culture of a whole embryo of unknown gestational age (Rasheed and Gardner, 1980). It supports the growth of all FeLV subgroups. AH927 cells were obtained from Dr. B. Willett (Department of

Veterinary Pathology, University of Glasgow) and were maintained in DMEM with added 10% FCS.

(2) QN10S

This is a feline cell line which was derived from the AH927 fibroblast cell line by Professor O. Jarrett. It carries the proviral DNA of the Moloney murine sarcoma virus containing the *mos* oncogene. It is therefore described as a S+ L- cell line. The cells are used for routine FeLV isolations in the diagnostic laboratory of the Feline Virus Unit, University of Glasgow. QN10S cells were obtained from the staff of the Feline Virus Unit and were maintained in DMEM with added 10% FCS and 25 mM HEPES. They were used between passages 60 and 90.

(3) F422

This is an FeLV positive feline cell line derived from a thymic lymphosarcoma (Rickard *et al.,* 1969). The cells produce FeLV of subgroup A. The F422 cell line was maintained in RPMI 1860 with added 10% FCS. The principle use of this cell line was as a source of purified FeLV. Large quantities of the cells were grown in roller flasks and the viral particles from the culture fluid were concentrated by ultrafiltration followed by centrifugation in a sucrose gradient. Preparations of virions purified in this manner were obtained from Mr. M. Golder. The virus was lysed with 1% Triton X100 on ice for 30 minutes, diluted with an equal volume of TBS, and stored at -20^{0}C until used.

(4) FL74

This is a transformed feline lymphoblastoid cell line which produces FeLV of all three (A, B and C) subgroups (Kawakami *et al.,* 1967). The FL74 cell line was maintained in RPMI 1860 with added 10% FCS. Concentrated virion preparations prepared in a similar fashion to that described above were obtained from Mr. M. Golder. Triton lysis was also performed on these preparations.

(5) 3201 B

This is a CD4 + / CD8 + feline T cell line derived from a naturally acquired, FeLV negative, lymphosarcoma (Snyder *et al.,* 1978). The cell line was obtained from Dr. B. Willett, and maintained in RPMI 1860 with added 10% FCS.

This cell line is sometimes also known as FeT (Kristal *et al.,* 1993). It is reported to support the growth of FeLV-A and -C but not FeLV-B, albeit at a much lower level than that seen with AH927. FeLV-C has been reported to induce apoptosis in these cells when infected at a high multiplicity of infection (Rojko *et al.,* 1991).

(6) MDCK

This is a canine fibroblast cell line derived from the kidney of a normal adult cocker spaniel which supports the growth of a wide range of mammalian viruses (Madin *et al.,* 1957). MDCK cells were obtained from Dr. N. Spibey (Department of Veterinary Pathology, University of Glasgow), and were maintained in DMEM with added 10% FCS. The ability of MDCK cells to support the growth of FeLV was not known at the start of this project.

(7) HuT 78

This is a human T cell line derived from a cutaneous lymphoma of a patient suffering from Sezary Syndrome (Gazdar *et al.,* 1980). The tumour cells were induced to proliferate *in vitro* using the mitogen concanavalin A. They are mature T cells of the helper phenotype, expressing the CD4 antigen and constitutively producing IL-2. They are susceptible to HIV infection. They are free from HTLV-1 infection (Mann *et al.,* 1989). Their susceptibility to FeLV infection was unknown at the start of this project.

The cell line was obtained from Dr. M. Harris (Department of Veterinary Pathology, University of Glasgow), and maintained in RPMI 1860 with added 10% FCS.

(8) Sf9

The Sf9 cell line, a subclone of Sf21, was derived from the ovarian tissue of the fall army leaf worm (*Spodoptera frugeriperda*) (Vaughn *et al.,* 1977). These cells were used throughout the project for the production of recombinant viruses and proteins. The cells were obtained from Dr. M. Harris.

Sf9 cells were maintained in TC100 media with added 10% FCS at 28°C without CO_2 supplementation. For the production of recombinant proteins the cells were maintained in SF900 media, a serum-free insect cell medium, after infection with the recombinant virus. A much improved version of the SF900 medium, SF900 II, was used when it became available. No serum-free medium was found to be able to support the growth of insect cells for more than 3 passages.

2.2 Manipulation of DNA

Most DNA manipulations were carried out using standard published protocols (Sambrook *et al.* 1989) with commercially available enzymes and buffers.

2.2.1 Preparation of Plasmid DNA

To generate sufficient quantities of DNA for use in transfections and enzymic manipulations a Triton X100 (TX100) lysis technique was used.

A 500 ml bacterial suspension, cultured overnight, was pelleted using a JA14 rotor in a J2-21 centrifuge at 6,000 rpm for 15 minutes. The supernatant was discarded and each pellet was resuspended in 2 ml of 12.5% sucrose in 50 mM Tris (pH 8.0). The suspensions were pooled in JA20 centrifuge tubes (10 ml per tube) and 1 ml of freshly prepared lysozyme solution (10 mg/ml) was added. After 5 minutes 1 ml 0.5 M EDTA was added rapidly, followed by a solution of 2% TX100 in 50 mM Tris (pH 8.0) with 62 mM EDTA (1 ml/ml suspension). The suspension was kept on ice for a further 15 minutes.

The lysed bacterial debris was pelleted by centrifugation at 15,000 rpm for 60 minutes. For every 1 ml of supernatant so formed, 1 g of caesium chloride was added, together with ethidium bromide solution (100 µl / 10 ml solution). The solution was then pipetted into polypropylene tubes and centrifuged at 55,000 rpm overnight using a VTi 65 rotor. The DNA was visualised under UV light and was harvested using a hypodermic needle and syringe. The ethidium bromide was removed using butanol extraction and the DNA was ethanol precipitated, dried and dissolved in sterile water. The quantity of DNA was measured using a Beckman DU 64 spectrophotometer in scan mode. The quantity of plasmid DNA was estimated using the conversion of OD_{260} = 1.0 equivalent to 50 µg/ml. The preparations routinely had an OD_{260}/OD_{280} ratio of approximately 2, indicating that they were free from contaminating proteins.

2.2.2 Phenol/Chloroform Extraction of DNA

To remove proteins from DNA solutions an equal volume of phenol was mixed with the DNA solution and the two phases allowed to partition. The aqueous phase was removed and an equal volume chloroform was added to it. The two phases were allowed to separate and the aqueous component was retained. These procedures were performed in a fume hood.

2.2.3 Ethanol Precipitation of DNA

DNA was purified from solutions by adding 2.5 volumes of pure ethanol and 0.1 volumes of sterile 5 M NaCl. The precipitate was allowed to form for 1 hour at -20°C and then pelleted in a benchtop 'Microcentaur' centrifuge (M.S.E.) at 13,000 rpm for 15 minutes. The pellet was washed twice in 70% ethanol before drying by centrifugation under vacuum in an 'Univap' evaporator (Uniscience Limited).

2.2.4 Restriction Enzyme Digestion of DNA

Commercially available restriction enzymes were used with their appropriate buffers. When double digests required different buffers, the DNA was ethanol precipitated between digests. Generally 2 units of enzyme were used to digest 10 μg of DNA.

2.2.5 Agarose Gel Electrophoresis

Separation of DNA of differing sizes was achieved by electrophoresis through a 1% agarose gel made with TBE buffer. The gel was made with a small quantity of ethidium bromide (7 μl stock solution / 100 ml gel). The DNA solutions were mixed with agarose gel loading buffer prior to loading. During electrophoresis the gel was examined periodically under UV light until the desired separation of the products was achieved.

The molecular mass markers used with agarose gels were derived from λ DNA digested with *Hind* lll or ΦX RF DNA digested with *Hae* lll (Gibco).

2.2.6 Purification of DNA from Agarose

A standard method of electroelution was used for most purifications of DNA from agarose (Sambrook *et al.* 1989). A slab of agarose containing the DNA was placed in a dialysis bag with a small volume of sterile buffer and the bag was placed in the electrophoresis chamber. Voltages of 100 V over a period of 3-4 hours were used and allowed good recovery. After electroelution the DNA was precipitated with ethanol.

For fragments of DNA less than 1 kbp, the slabs of agarose were repeatedly frozen and thawed in SPIN-X (Costar) tubes containing 0.22 μm cellulose acetate filters and were then centrifuged in a bench top centrifuge at 13,000 rpm for 20 minutes.

2.2.7 Acrylamide Gel Electrophoresis of DNA

To visualise small (less than 0.5 kbp) fragments of DNA, electrophoresis through a 7.5% acrylamide gel was used. These gels were made with TBE buffer and polymerised with TEMED (1.5 μl/ml gel) and 10% ammonium persulphate (6 μl/ml gel). After electrophoresis the gels were stained with fresh buffer containing a small quantity of ethidium bromide. The DNA solutions were mixed with agarose gel loading buffer prior to loading.

The molecular mass markers used with acrylamide gels were derived from ΦX RF DNA digested with *Hae* lll (Gibco).

2.2.8 Purification of Synthetic Oligonucleotides

Following synthesis in a 381A DNA Synthesizer (Applied Biosystems) the oligonucleotide (without a terminal trityl group) was eluted from the column with 30% ammonia and then deprotected by incubation overnight in a water bath at 55°C. The following day the solution was cooled, then precipitated with ethanol. Aliquots of the oligonucleotides were stored at -20°C. The quantity of oligonucleotide was estimated using a spectrophotometer and the conversion of OD_{260} = 1.0 equivalent to 35 μg/ml.

2.2.9 Amplification of DNA by PCR

A commercial kit obtained from Boehringer Mannheim was used for all polymerase chain reaction (PCR) manipulations. This contained the *Taq* DNA polymerase as well as the reaction buffer and dNTPs. The kit was used according to the manufacturer's instructions. Reaction mixes containing the appropriate concentration of buffer, enzyme and 200 nmoles of each dNTP were dispensed in aliquots prior to the addition of primers and templates. For each 100 μl of reaction mix 1 μg of each primer was added and 1 μl of template stock (approximate concentration 1 ng/μl). The reaction mixes had 50 μl of mineral oil placed on top to reduce evaporation losses. The reactions were performed in an air cooled Omnigene TR3 thermal cycler (HyBaid). Reaction parameters are given in the appropriate chapters. Temperature changes were effected as rapidly as possible. Preparative yields of product were prepared from several reaction aliquots utilising the original template. PCR products were extracted with phenol/chloroform and then ethanol precipitated before digestion with restriction enzymes.

2.2.10 Sequencing of DNA

Sequencing of plasmid DNA was carried out using a Sequenase kit (Stratagene) and ^{35}S dATP (Amersham International) for dideoxy-mediated chain termination method (Sanger *et al.,* 1977)). The manufacturer's instructions for the composition of the sequencing reactions were followed and the 6% polyacrylamide gel with 8 M urea was prepared in a Sequi-gen nucleic acid sequencing cell (Bio-Rad) as detailed elsewhere (Sambrook *et al.* 1989). After prewarming the gel for 1 hour at 60 W the gel was loaded and electrophoresis continued until the either the xylene cyanol or bromophenol blue had reached the bottom of the gel. This generally took 2 to 4 hours and allowed about 300 bp to be sequenced. The glass plates were then separated and the gel was removed using a large sheet of Whatman 3M paper. The gel was then dried under vacuum in a Model 583 gel dryer (BioRad, California). Once the gel was dry to the touch an autoradiograph was prepared. Sequencing was performed on both strands of DNA using two sets of reactions, employing different

primers.

2.3 Manipulation of Proteins

2.3.1 Determination of Total Protein Content

This was performed by a microtitre assay using Bradford's reagent (Dunn, 1989). This solution was made by adding 100 mg Coomassie Blue G250 and 30 mg SDS to 50 ml 95% ethanol and then adding 100 ml of 85% phosphoric acid. The final volume was adjusted to 200 ml and the resulting solution used as a 5 x stock (Sambrook *et al.* 1989).

A volume of 100 µl of the diluted reagent was added to 10 µl of protein solution and was incubated at room temperature for 10 minutes. The OD_{595} was then measured in an EL312 Bio-Kinetics Plate Reader (Bio-Tek Instruments). A standard curve using bovine albumin was generated with each assay which was repeated at least twice for each sample. This test works well for low protein concentrations (0.05 mg/ml - 1 mg/ml).

2.3.2 Polyacrylamide Gel Electrophoresis of Proteins

For the routine separation of proteins, electrophoresis through a polyacrylamide gel with a discontinuous buffer system containing SDS was used (Laemmli, 1970). Normally a 7.5% polyacrylamide separating SDS-PAGE gel was overlaid with a 4% polyacrylamide stacking gel. Gradient gels were made with a Multiphor II gradient maker (Pharmacia). Gels were made by adding TEMED (1.5 µl/ml gel) and 10% ammonium persulphate (6.0 µl/ml gel) to freshly prepared solutions containing diluted acrylamide/bis-acrylamide mixture and the appropriate buffer. Normally a MiniProtean (Bio-Rad) gel apparatus was used. When larger glass plates were used, a very thin film of petroleum jelly was used to coat the spacer bars to ensure a good seal. Prior to loading, the samples were boiled with SDS-PAGE gel loading buffer. Electrophoresis was continued until adequate resolution of the Rainbow Markers (Amersham International) has been achieved.

2.3.3 Staining SDS-PAGE Gels

(1) Coomassie staining

For routine staining of SDS-PAGE gels a solution of Coomassie brilliant blue R250 in 50% methanol, 45% water and 5% acetic acid was used followed by destaining in 50% methanol, 45% water and 5% acetic acid. Using this technique 0.5 µg of protein could be visualised.

(2) Silver staining

For the sensitive detection of small quantities of proteins a silver staining technique similar to that described elsewhere (Sambrook *et al.* 1989) was employed.

The gels were initially fixed for 4 hours in 30% ethanol, 60% water and 10% acetic acid and were then incubated for 1 hour in 30% ethanol in water. Rehydration using deionised water for 2 hours was followed by 30 minutes in a 0.1 μg/ml solution of DTT in deionised water. The gels were stained with a 0.1% solution of silver nitrate (freshly prepared from a 20% stock) for 30 minutes and were then washed three times with deionised water. The staining was carefully developed with a freshly prepared solution of sodium carbonate in formaldehyde. Once sufficient intensity had been achieved the reaction was stopped using 1% acetic acid. The gels were then washed thoroughly in deionised water and preserved (see below).

Using this technique 0.05 μg of protein could be visualised.

2.3.4 Preserving SDS-Polyacrylamide Gels

Polyacrylamide gels were preserved by enclosing in a moist cellophane sandwich which was then baked in an Easi-Breeze gel dryer (Hoefer Scientific Instruments).

2.3.5 Western Blotting

Following SDS-PAGE to separate the polypeptides of a protein mixture, detection by immunological reactivity was achieved by western blotting (Towbin *et al.*, 1979).

Gloves were worn at all times during this procedure to ensure that skin oils did not prevent efficient protein binding. A plastic cassette with two foam pads was used to hold the membrane and gel together. Two sheets of Whatman 3M filter paper were cut to the same size as the foam pads. One sheet of either 0.2 μm nitrocellulose 'Trans-Blot' membrane (BioRad) or 0.45 μm nylon 'HyBond' membrane (Amersham International) membrane was cut to just a little larger than the polyacrylamide gel. The membrane was then soaked in western buffer. After SDS-PAGE to separate the polypeptides, one sheet of filter paper was used to transfer the polyacrylamide gel off the glass plate and on one to the foam pads on the black side of the opened cassette. The membrane was carefully placed on top and the second sheet of filter paper (soaked in western buffer) was placed on top. Air bubbles were removed by rolling a pipette over the top of the sandwich. The second foam pad was then placed on top and the cassette closed and placed in the chamber with the gel (black) side of the cassette nearest to the negative terminal of the chamber. Electroblotting of the proteins from the gel to the membrane was performed at 120 V for 1 hour

(small gels) or 180 V for 1.5 hours (large gels). The apparatus was kept cool using an ice block and the western buffer was circulated using a magnetic stirrer.

When electroblotting was complete the membrane was removed from the apparatus and placed in a clean container containing the blocking solution (see section 2.1.3) and gently rocked for 4-12 hours. If a goat polyclonal anti-antibody preparation was to be used then 10% goat serum was added to the blocking solution. The membrane was then washed several times using PBS-Tween (see section 2.1.3) over a period of 2 hours.

The blocking solution had 0.2%(v/v) 0.5 M EDTA, 0.5%(v/v) Tween 20 and the appropriate quantity of serum / monoclonal antibody added. The solution was then incubated with the membrane in a plastic bag on a rapidly rotating platform for 2 hours. Care was taken to exclude any air bubbles from the bag. If a second antibody was to be used then the membrane was briefly washed in PBS-Tween before incubating with a similarly prepared solution for a further hour. All such antibodies were conjugated with alkaline phosphatase.

The membrane was then washed several times using PBS-Tween over a period of 1 hour. The membrane was finally washed in alkaline phosphatase (AP) buffer. The blots were developed by incubating with a substrate solution containing 1.65 μg BCIP and 3.3 μg NBT in 10 ml of AP buffer. The reaction was allowed to proceed until the purple staining bands could be clearly seen. The reaction was stopped with water or Tris-EDTA and the membrane was photographed promptly.

2.3.6 ELISAs

Several different enzyme linked immunoabsorbant assays (ELISAs) (Belanger *et al.*, 1973) were performed. The general method that was used is described below. Specific details are given in the relevant chapters.

A positive and a negative control were always included. All reactions were performed at room temperature with gentle agitation between stages. An ELISA plate washer (Ultrawash II; Dynatech) was sometimes used.

A 96-well, medium binding, flat-bottomed microtitre plate (Greiner) was coated with an antibody preparation diluted with coupling buffer (100 μl per well) and left overnight. The plate was washed twice with TBS-Tween (see section 2.1.3) and was then blocked for 1 hour with blocking solution (200 μl per well). The plate was washed twice more with TBS-Tween. The antigens to be tested were diluted using 1% empigen in TBS and then added to the plate (100 μl per well) for 3 hours. A further 2 washes were followed by the addition to each well of 100 μl of the second

antibody preparation, also diluted in blocking solution with 0.5% Tween 20 added. The plate was then incubated for 1 hour, washed twice and an alkaline phosphatase linked anti-antibody preparation (diluted in blocking solution with 0.5% Tween 20) was added. The plate was then washed at least 6 times with TBS-Tween before adding 50 µl of a 1 mg/ml solution of 4-nitrophenyl phosphate in AP buffer to each well and incubating for approximately 4 hours.

The reaction was stopped with 50 µl of 0.4 M NaOH and the result was read at 405 nm using an EL312 Bio-Kinetics Plate Reader (Bio-Tek Instruments).

2.4 Growth and Manipulation of Prokaryotic cells

2.4.1 Growth of Bacteria

Small colonies of bacteria were grown on L agar plates, containing the appropriate antibiotic. When necessary these plates were impregnated with X Gal by carefully spreading out 100 µl of X Gal stock solution and leaving to dry at 37°C.

Large scale cultures of DS941 bacteria were grown in an orbital shaker at 37°C using L broth supplemented with the appropriate antibiotics. This required 25 µg streptomycin per millilitre of culture.

2.4.2 Storage of Bacteria

For long term storage of bacteria 0.5 ml of an overnight culture were added to 0.5 ml filter sterilised 30% glycerol and immediately frozen to -70°C.

2.4.3 Transformation of Bacteria

Transformations of bacteria were performed by electroporation using competent cells.

(1) Preparation of competent cells

The recipient bacterium for all electroporations was *E.coli*, strain DS941. These cells were initially prepared for electroporation by adding a 10 ml overnight culture to 1 litre of L Broth and growing to mid logarithmic phase (at about an OD_{600} of 0.6). This took about 2 hours. The cells were then pelleted at 5,000 rpm for 15 minutes using a JA14 rotor in a J2-21. This washing procedure was repeated several times. At each subsequent washing step the cells were resuspended in a smaller volume. The final wash used ice-cold 10% glycerol and the cells were then resuspended in 2 ml of 10% glycerol and aliquots of 100 µl were frozen to -70°C. The cells remained competent for several months using this procedure.

(2) Electroporation

Prior to electroporation 40 µl of competent bacteria were placed in ice cold, sterile, cuvettes with electrodes set 2 mm apart. An aliquot of 2 µl containing approximately 40 ng of the DNA to be electroporated was then introduced. Care was taken to ensure that the DNA solution was free from salts. This was achieved by ethanol precipitating all DNA solutions before electroporation. The DNA was left with the bacteria in the cuvettes for about 2 minutes.

To electroporate the bacteria, a Gene Pulsar (Bio-Rad) set at 2.5 kV, 25 µF and 200 Ω was used. These parameters allowed the delivery of a pulse of approximately 3.9 msec duration. After the pulse the cells were resuspended as rapidly as possible in 1 ml of L Broth containing ampicillin (100 µg/ml) and were then incubated for 1 hour in an orbital incubator set at 37°C. The cells were pelleted and resuspended in 0.5 ml of fresh L Broth. This suspension was spread out on an agar plate containing a selective antibiotic and, if appropriate, X-Gal.

(3) Identification of recombinants

To screen transformed bacteria for the presence of recombinant plasmids, colonies were picked from the agar plates and spread out in small patches on another L agar plate impregnated with ampicillin (100 µg/ml) and streptomycin (25 µg/ml). This plate was then incubated at 37°C overnight. The patches were scraped off with sterile toothpicks and resuspended in 100 µl of STET solution (see Standard Solutions above). A 10 µl volume of lysozyme solution (10 mg/ml) was added to each and the suspensions were left for 15 minutes at room temperature before being plunged into boiling water for exactly 40 seconds. The chromosomal DNA and associated protein were pelleted by centrifugation in a benchtop centrifuge at 15,000 rpm for 15 minutes. The supernatant was removed carefully and 10 µl was analysed by agarose gel electrophoresis. The remainder of the supernatant was diluted with 3 volumes of water, then ethanol precipitated for restriction enzyme digests.

2.5 Growth and Manipulation of Eukaryotic cells

2.5.1 QN10S cells

The cells, which attach firmly to plastic, were subcultured by carefully washing once in PBS-EDTA and then once in 0.025% trypsin in PBS-EDTA before adding more trypsin / PBS-EDTA (5 ml per 75 cm^2 tissue culture flask). They were seeded for routine maintenance at 1 x 10^6 per 75 cm^2 flask and were subcultured twice a week.

Infections of QN10S cells with FeLV were performed using 12-well tissue culture trays containing 22 mm diameter wells. The cells were subcultured the day previously and seeded at a density of 4 x 10^4 per well. The virus was inoculated into 1 ml of cell supernatant enriched with 4 μg/ml polybrene. The cells were incubated at 37°C for 2 hours with occasional agitation and the inoculum was then replaced with fresh medium. Foci of transformed cells were usually visible 5 days post infection. To ensure accurate estimation of foci numbers the foci were counted microscopically 6 days post infection. The plates were drained of their medium 7 days post infection and the cells fixed and stained in 0.5% (w/v) crystal violet in 4% formaldehyde. The plaques were then visible to the naked eye and were recounted. Occasionally this stage was not possible as the plaques were too large to be distinguished.

2.5.2 Sf9 cells

These cells, which only loosely adhere to plastic, were subcultured by removing the medium and gently scraping the cells into fresh medium with a plastic scraper. For general maintenance, 5 x 10^6 cells were inoculated into a 75 cm^2 tissue culture flask. The cells were subcultured on the day they became confluent to prevent them from becoming overgrown and detaching from the plastic surface. The cells required subculturing every 3 days or so using this density.

For transfections and infections the following densities were seeded the previous day: 1 x 10^6 per 25 cm^2 flask; 3 x 10^6 per 75 cm^2 flask; 4 x 10^5 per well of a 6-well dish. These densities gave a thin covering of cells that allowed at least 4 days of subsequent growth. After overnight incubation the medium was gently removed and a small volume of diluted virus (e.g 5 ml in a 75 cm^2 flask) was inoculated and incubated for 2 hours at 27°C before replacing with fresh medium. For maximal virus production 0.1 plaque forming unit per cell was inoculated.

To assay for β galactosidase activity in cells the supernatant was removed and the cells were fixed in a solution containing 2% formaldehyde and 0.2% glutaraldehyde in PBS for 10 minutes at 4°C. They were then washed twice before staining for 30 minutes with 50 mM potassium ferricyanide, 50 mM potassium ferrocyanide, 20 mM $MgCl_2$ and 0.4 mg/ml X-Gal in PBS.

(1) Transfection of plasmid DNA

A standard calcium phosphate precipitation technique (Sambrook *et al.* 1989) was found to be effective for all transfections involving Sf9 cells. Approximately 20 μg of plasmid DNA and 1 μg of wild type AcMNPV DNA were added to a $CaCl_2$ solution to make a solution with a final volume of 0.5 ml and a $CaCl_2$ concentration

of 250 mM. A volume of 0.5 ml of 2 x HEPES buffered saline was then added drop by drop. A faint cloudy precipitate normally developed over a period of 30 minutes. After this time the suspension was added, with 5 ml of fresh medium, to a 75 cm^2 flask of cells in the mid-logarithmic phase of growth. The cells were incubated for 2 hours and then were gently washed with medium. The cells were then maintained in fresh medium and examined daily for the presence of polyhedrin.

2.6 Growth and Manipulation of Viruses

2.6.1 FeLV

To ease the handling of FeLV, aliquots of high titre FeLV stocks were diluted 1 part in 10 with fresh medium and stored at -70°C. The diluted stocks were then titrated once, using QN10S cells, and then at regular intervals afterwards. No decrease in titre was observed in viruses so handled. Once thawed the aliquots of the diluted virus were discarded after use.

2.6.2 AcMNPV

Most of the techniques used in course of this work are similar to those described in a recently published account of the general laboratory handling of baculoviruses (King and Possee, 1992).

To prepare a stock of AcMNPV (either wild type or recombinant) a confluent 225 cm^2 flask of Sf9 cells (which contains about 6 x 10^7 Sf9 cells) was subcultured into 8 similar flasks and the cells were grown to approximately 60% confluence, before infecting with 0.1 pfu AcMNPV per cell. The flasks were incubated for 5-7 days at 27°C before harvesting the cells and supernatant into 50 ml polypropylene tubes. The supernatant was clarified by centrifugation at 3500 rpm for 10 minutes in a J2-21 and was then stored at 4°C. Most virus stocks were not filtered.

All baculoviruses were routinely stored at 4°C. The stocks were then titrated once, using Sf9 cells in a 6-well tissue culture flask without an agarose overlay, and then at regular intervals afterwards. A decrease in titre of approximately five-fold was observed over a period of 6 months with viruses stored in this manner. For long-term storage, concentrated stocks (see below) of the viruses were frozen at -70°C. Storage of unconcentrated stocks in this manner resulted in 1,000-fold decrease in titre. The lack of a convenient method of long term storage is a minor disadvantage of this system. In general the titres achieved by this route were the same as those published (approximately 1 x 10^7 to 5 x 10^7) (King and Possee, 1992).

(1) Manufacture of concentrated AcMNPV stocks

Concentrated AcMNPV stocks are required for the production of DNA and long-term storage of the virus. To prepare these stocks the virus was pelleted from 200 ml of virus stock with a titre of greater than 10^7 pfu/ml by centrifugation at 20,000 rpm for 1.5 hours using JA 20 rotor. The pellets were soaked overnight in a small volume of TE.

The next day, the virus was resuspended by vigorous pipetting and the resulting suspension was layered onto a sucrose gradient formed by pipetting 3 ml of 10% sucrose in TE onto a 2 ml cushion of 50% sucrose. The virus was purified by centrifugation at 24,000 rpm for 1 hour using a SW40 rotor. The thin translucent layer of viral particles at the interface of the sucrose layers was harvested with a hypodermic needle and syringe using strong overhead illumination.

The virus was then diluted 1 : 5 with TE and pelleted at 30,000 for 1 hour in a SW50 rotor. The viral pellet was then soaked overnight in TE and resuspended the next day. The concentrated viral suspensions were stored at 4^0C.

(2) Preparation of AcMNPV DNA

To prepare AcMNPV DNA for transfection about 200 µl of the purified virus was incubated with 200 µl TE and 100 µl 20% sarkosyl in TE at 60^0C for 30 minutes. The reaction mix was then layered on top of a solution of 54% caesium chloride with 25 µg/ml ethidium bromide in polycarbonate centrifuge tubes for the SW50 rotor. The DNA was separated from the viral proteins by centrifugation at 35,000 rpm overnight at 20^0C. 20 µg of AcMNPV transfer vector was used as a control. The band of viral DNA was visualised and harvested under UV light. Butanol was used to remove the ethidium bromide followed by overnight dialysis to remove the caesium chloride. The quantity and quality of DNA was measured by spectrophotometry at 280 nm and 260 nm.

To prepare AcMNPV DNA for PCR, 10 µl of concentrated stock was lysed by plunging into a bath of boiling water with 90 µl TE for 4 minutes. These preparations were used as 10,000 x stocks for use as PCR templates.

Initial difficulties were encountered in the preparation of viral DNA for transfection. By allowing the cells to become 60% confluent and infecting with a relatively high multiplicity of infection, higher titres were achieved which allowed the production of the DNA. From eight 175 cm^2 tissue culture flasks approximately 20 µg of AcMNPV DNA was isolated.

(3) Plaque purification

Several methods have been proposed to purify recombinant from wild type baculoviruses. Several attempts using a limiting dilution protocol were all unsuccessful and the remainder of the purifications were achieved with a modification version of the agarose overlay technique (Brown and Faulkner, 1977).

To perform these purification steps the Sf9 cells were seeded at a density of 5 x 10^5 cells per well of a 6-well dish and incubated overnight. The following day the cells were infected with serial tenfold dilutions of the virus. Whilst the cells were being infected, a 1.5% low melting point agarose gel was made by melting 3 g low melting point agarose in 100 ml PBS, cooling to 37^{0}C and adding an equal volume of prewarmed medium. The virus inoculum was removed after 2 hours and 1.5 ml of the gel was added and allowed to cool. Before adding the gel the temperature was carefully monitored to ensure that it was less than 32^{0}C. After the gel had solidified a further 1.5 ml of fresh medium was added. As the normal X-Gal staining technique was not applicable to these assays, after 2 days the liquid medium was carefully replaced with fresh medium containing 80 µg/ml of X-Gal. For the following 2 days the wells were carefully examined for the development of blue plaques which were then examined microscopically. Those which contained the least polyhedrin positive cells were picked by removing the medium and carefully sucking up a plug of agarose and the underlying medium using a pastette. The plug was then introduced into 1 ml of fresh medium and was used as the stock for the next round of purification.

Chapter 3. Production of Recombinant Viruses

3.1 Introduction

The major aim of this project was to produce recombinant baculoviruses which expressed SU of FeLV subgroups A, B and C. The optimal expression of a gene in the baculovirus system requires the production of a stock of pure recombinant virus. This chapter details the generation and analysis of recombinant baculoviruses containing sequences coding for these FeLV SUs.

The transfer vector used in this system contained two promoters active in insect cells. The p10 promoter was employed to generate a selectable marker, β galactosidase, and the polyhedrin promoter was used to produce the FeLV SUs. A series of preliminary experiments were performed to establish if functional FeLV-A SU could be produced in the baculovirus system using this multiple expression vector. On the basis of the results, fragments of each SU were generated by PCR and then were inserted into a modified transfer vector. The resulting recombinant viruses were examined for the presence of the correct SU coding sequence using PCR and restriction enzyme analyses.

The production of a recombinant baculovirus which served as a negative control is also described. This baculovirus contained the sequences coding for the SU portion of the feline immunodeficiency virus (FIV) *env* gene. As the virus was made using the same transfer vector it also contained the *lacZ* gene for generation of β galactosidase.

3.2 Preliminary Experiments

Preliminary experiments were performed to investigate the ability of the baculovirus expression system to produce FeLV-A SU. No attempt will be made here to detail all the techniques used as they will be discussed at length below. Experiments which utilised other expression systems will also be briefly mentioned.

3.2.1 Baculovirus Expression System

Initially, all of the FeLV *env* gene was subcloned as a *Pst* 1 fragment from pFGA (Stewart *et al.,* 1986) into the pBluescript vector by Dr. N. Spibey. The resulting plasmid, called pBMA*env* (see Figure 3.4), was analysed using restriction enzymes. From this plasmid, all of the FeLV-A SU coding sequence and the non-anchoring portion of TM was subcloned as a *Bam* Hl / *Apa* 1 (blunted) fragment into a preparation of *Bam* Hl / *Sna* Bl digested pAcLacZ+ vector. The recombinant pAcLacZ+ vector (pAcA9) was co-transfected with wild type baculovirus DNA

into Sf9 cells. The supernatant was harvested 4 days later. The cells were fixed and then were stained with X-Gal, as described in Chapter 2. This demonstrated that recombinant viruses had been produced. A sandwich ELISA (described in detail in section 3.3.3) demonstrated the presence of recombinant gp70 in the supernatant. The recombinant virus was designated AcA9.

Using a limiting dilution technique, many attempts were made to purify AcA9 with limited success. These results suggested either that AcA9 was dependent upon a wild type, helper virus, or that the purification technique was inefficient. Two consecutive plaque purifications using agarose overlays did not produce a helper independent stock. Conversations with Dr. R. Possee suggested that the deletion of the small segment between the *Bam* Hl and *Sna* Bl sites was likely to be of functional significance.

Despite these problems, a large stock of AcA9 virus was produced and was used to infect cells at a high multiplicity. Recombinant FeLV gp70 was demonstrated in the supernatant by western blot with the monoclonal 3-17AP. This result prompted attempts to generate helper independent, pure, recombinant baculovirus stocks.

3.2.2 Other Expression Systems

Two other expression systems were tried in tandem with the early baculovirus system experiments.

The pSVL transient expression vector (Pharmacia) contains the SV40 promoter and is reported to work well in COS cells (Templeton and Eckhart, 1984). A *Bam* Hl / *Tha* 1 fragment of FeLV *env* (which includes all of SU and 15 codons of TM) was inserted into the pSVL plasmid to form pT6. However, on transfection of pT6 into COS cells no product was detected in the supernatant. No further attempts at expression were made using this system.

Attempts were also made to express short fragments of FeLV *env* using the inducible prokaryotic vector, pGEX-2T (Smith and Johnson, 1988). This vector generates glutathione-S-transferase fusion proteins. Fragments corresponding to the sequences between nucleotide numbers 278 & 532 and 278 & 957 (Stewart *et al.*, 1986) were amplified by PCR. The fragments were then inserted into pGEX-2T. Fusion proteins were expressed, but in preliminary experiments did not appear to interfere with viral entry or with the action of neutralising antibodies.

3.3 Results

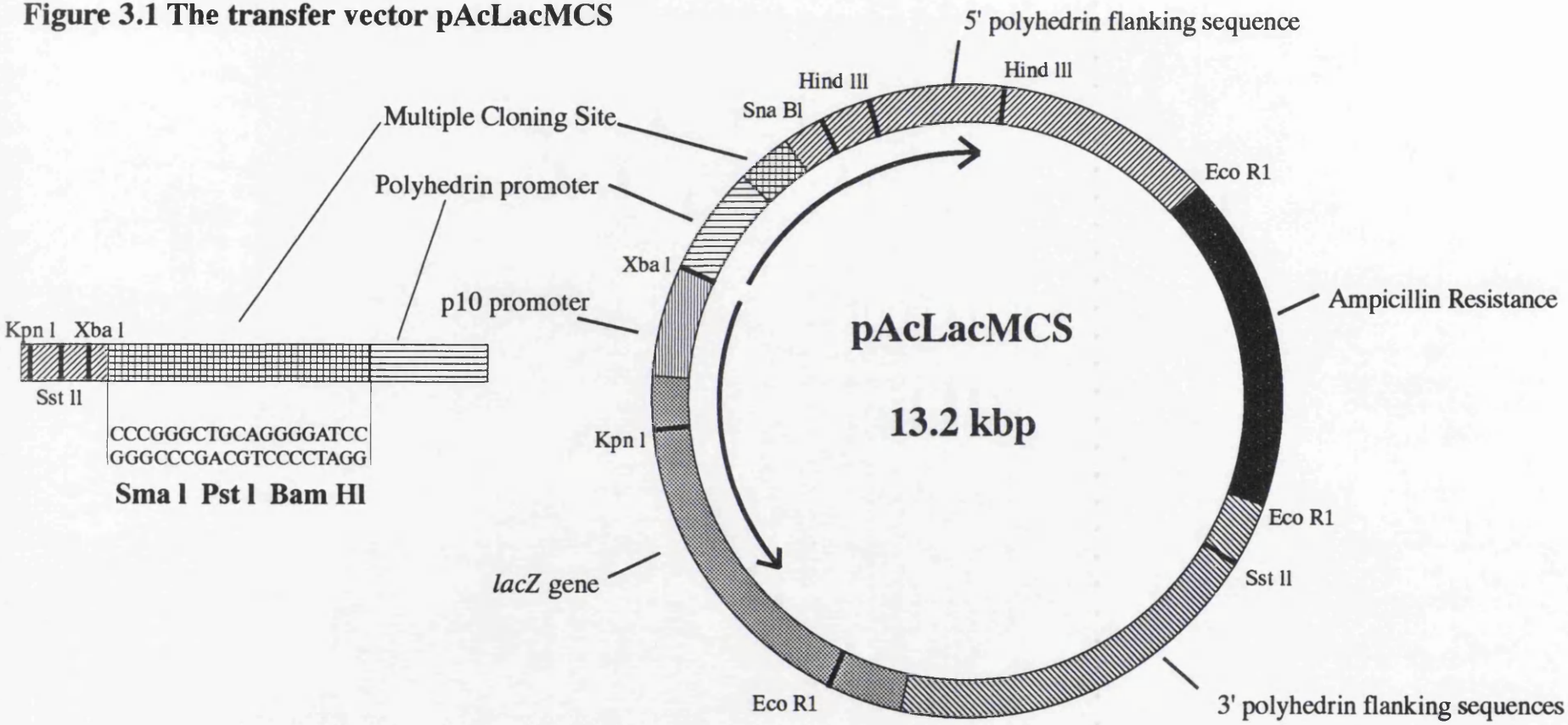
3.3.1 Manufacture of Recombinant pAcLacMCS Plasmid

The results from the preliminary experiments were sufficiently encouraging to suggest modification of pAcLacZ+ by the insertion of a multiple cloning site (MCS) at the *Bam* Hl site. Complimentary synthetic oligonucleotides, 5' GAT CCC TGC AGC CCG GGT CTA GAC CGC GGG GTA CCA 3' and 5' GAT CTG GTA CCC CGC GGT CTA GAC CCG GGC TGC AGG 3' were incubated at 90^{0}C for a period of 1 hour in Klenow buffer then allowed to cool to 40^{0}C over a period of 2 hours. The annealed products were purified by acrylamide gel electrophoresis and spin membrane centrifugation (SPIN-X, Costar). The products were ligated into a preparation of *Bam* Hl cut pAcLacZ+ which had been purified by agarose gel electrophoresis and electroelution. These oligonucleotides introduced *Pst* 1 and *Sma* 1 sites, as well as preserving the *Bam* Hl site. Additional, non-unique, sites were introduced for *Xba* 1, *Sst* 11 and *Kpn* 1 to allow easier restriction enzyme analysis of the resulting recombinant plasmids. Multiple copies of the MCS were removed by Dr. N. Spibey by digestion with *Pst* 1. The products from this reaction, after purification by agarose gel electrophoresis and electroelution, were then religated. The restriction enzyme sites of the resulting plasmid, designated pAcLacMCS (see Figure 3.1), were checked with an extensive selection of enzymes. A sample of these digests is presented in Figure 3.2.

To confirm that pAcLacMCS could produce pure recombinant virus a *Bam* Hl / *Apa* 1 (blunted) fragment of FeLV-A *env* was subcloned from pBMA*env* (see section 2.1 above and Figure 3.4), into a preparation of *Bam* Hl / *Sma* 1 digested pAcLacMCS. The resulting recombinant transfer vector, pAcA22, was co-transfected with wild type baculovirus DNA into Sf9 insect cells. The recombinant virus, termed AcA22, was purified from the supernatant using 5 rounds of a plaque purification technique (Brown and Faulkner, 1977) modified as described in Chapter 2. A titrated stock was then used to infect 10 large (225 cm^2) tissue culture flasks each containing 2 x 10^7 cells at about 30% confluence and a multiplicity of infection of 0.15. After infection the virus inoculum was removed and replaced with SF900 serum free medium. The supernatant was harvested 5 days later and was concentrated by ultrafiltration (see Chapter 4). Expression was demonstrated by several of the techniques, described in Chapter 4. Early experiments with neutralisation inhibition (Chapter 5) and infection interference (Chapter 6) assays were also performed, with promising results.

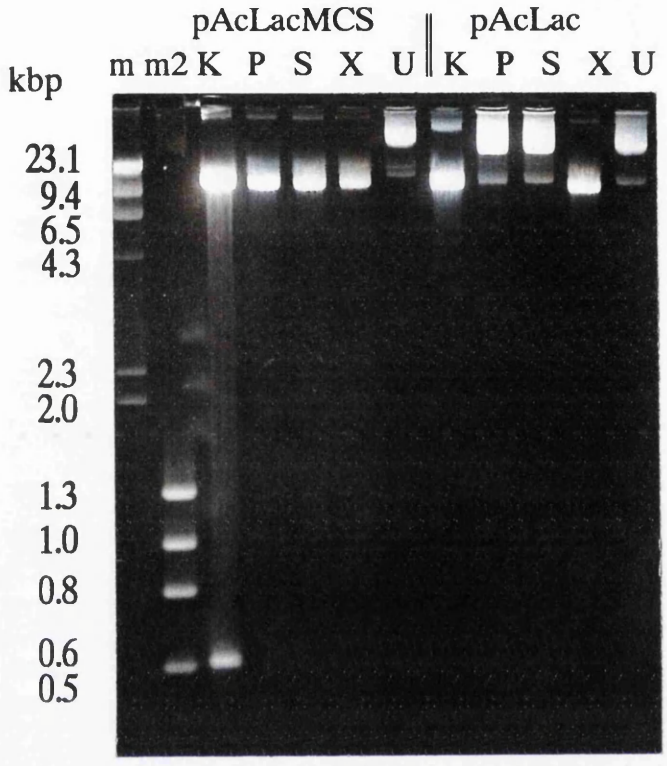
A similar cloning strategy was followed with a *Bam* Hl / *Sst* 11 fragment of FeLV *env* (which includes all of SU and 15 codons of TM). Several pure recombinant

Figure 3.1 The transfer vector pAcLacMCS



This diagram is a schematic representation of the baculovirus transfer vector pAcLacMCS. The arrows represent the direction of transcription There are two polyadenylation sequences contained within this plasmid; the first, derived from SV40 is contained at the end of the *lacZ* gene; the second is downstream of the cloning site. All the sites of the following restriction enzymes are shown; *Bam* HI, *Eco* RI, *Hind* II, *Kpn* I, *Pst* I, *Sma* I, *Sna* BI, *Sst* II and *Xba* I.

Figure 3.2 Restriction enzyme digests of the pAcLacMCS vector



To confirm the insertion of a multiple cloning site into the vector pAcLacZ+ an array of restriction enzyme digests was performed. Aliquots of 2 μg of each plasmid were incubated with 5 units of each enzyme in the appropriate buffer for a period of 2 hours. The products were separated by 1% agarose gel electrophoresis. The photograph is of the ethidium bromide stained gel.

Key

K = *Kpn* 1, P = *Pst* 1, S = *Sma* 1, X= *Xba* 1, U = Uncut, m/m2 = molecular mass markers

viruses were produced but no secreted SU was ever demonstrated in the supernatants of infected cells. No investigation was undertaken to determine the reason for this lack of expression.

On the basis of these experiments a stratagem was developed to express all 3 FeLV subgroup SUs using the pAcLacMCS transfer vector. It was decided that the SU fragments would be generated by PCR as the *env* gene of subgroups B and C do not possess convenient restriction sites. The use of this technique would also facilitate the inclusion of sequences conferring specific activity on the recombinant proteins and consequently would permit purification by exploitation of that activity. The addition of 6 histidine residues would allow the use of a nickel-nitrilotriacetic acid adsorbent with bound nickel to affinity purify the proteins from the supernatants of infected cells (Hochuli *et al.,* 1987). This adsorbent had previously worked well in the purification of recombinant HIV *nef* produced using the baculovirus system in the Department of Veterinary Pathology (Harris and Coates, 1993).

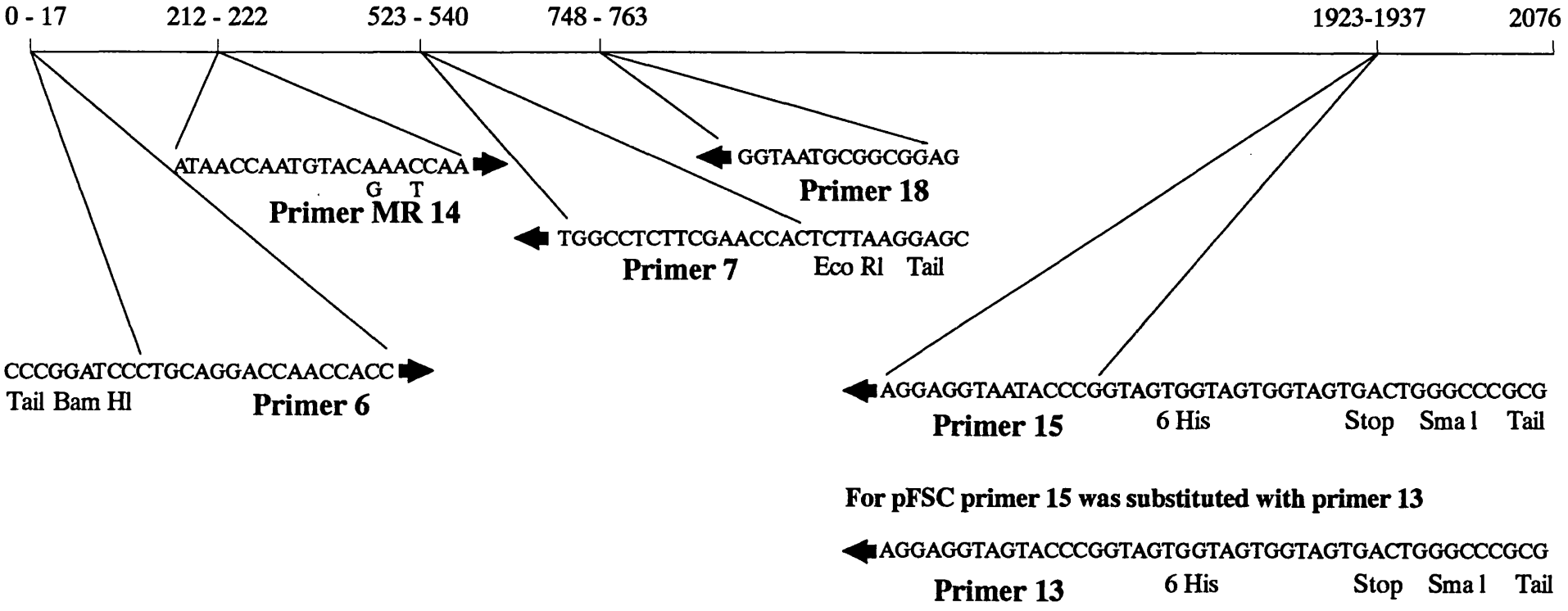
3.3.2 Manufacture of Recombinant pAcLacMCS Transfer Vectors

The oligonucleotides that were used in the PCR reactions described below are defined in Figure 3.3. The cloning strategy that was used is illustrated in Figure 3.4.

Preparative PCRs were performed with the following parameters; annealing, 55°C, 1 minute; extending 72°C, 5 minutes; denaturing, 90°C, 1 minute. The reactions were introduced into the thermal cycler once the recorded temperature reached 80°C. This hot start technique is believed to increase the specificity of the reaction (Erlich *et al.,* 1991). Each reaction was performed in quadruplicate. At the end of the reaction a 10 µl aliquot was analysed by agarose gel electrophoresis and the rest of the reactions were pooled, phenol extracted and then ethanol precipitated. The PCR products were digested, first with *Bam* HI, and then with *Sma* I. The digested products were then purified by agarose gel electrophoresis, followed by electroelution.

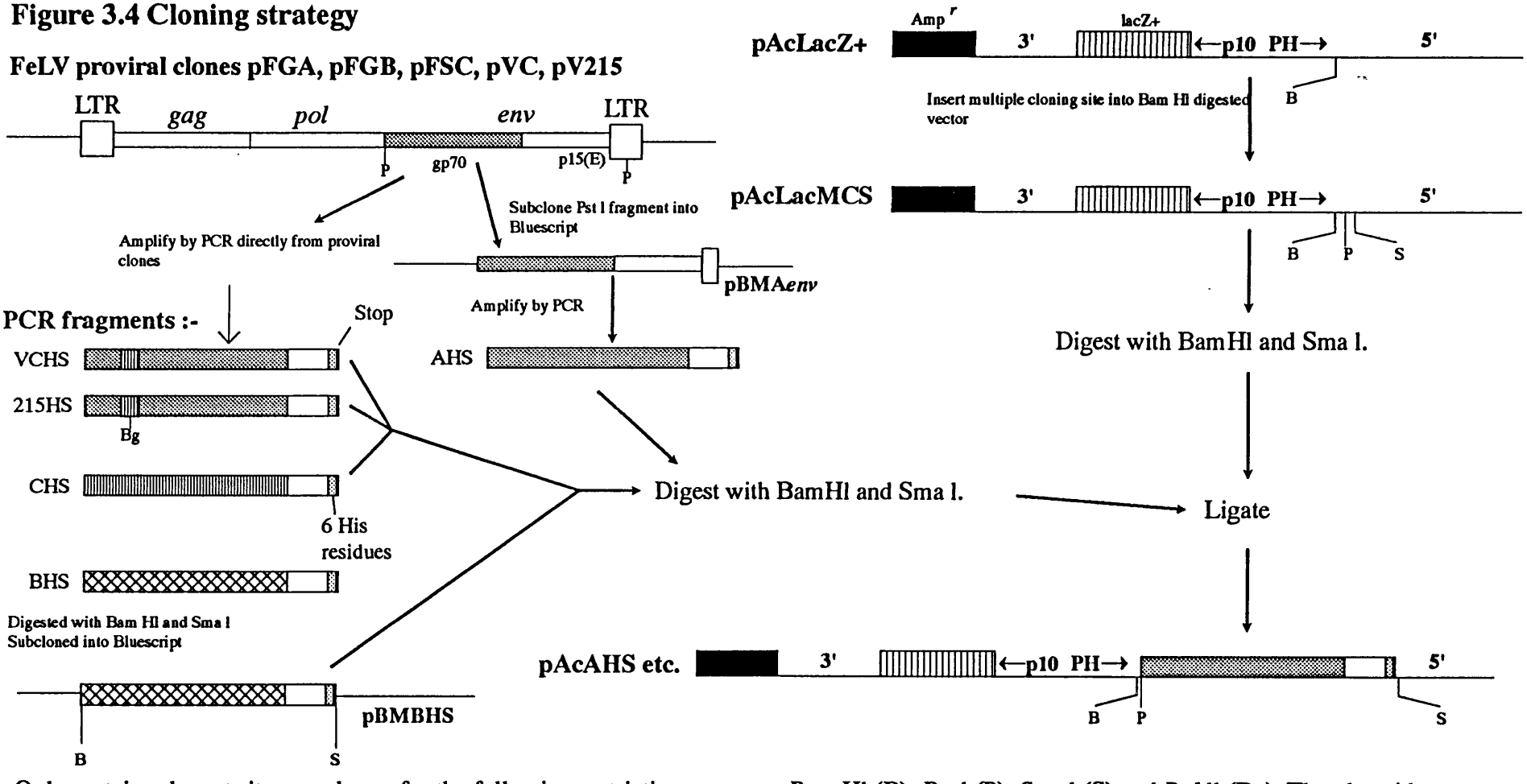
Using PCR as defined above, fragments of the *env* genes of the proviral clones pFGA, pFSC and two A/C chimeras pV215 and pVC (Rigby, 1989) were generated with 6 histidine residues and stop codons at their carboxy termini. These fragments included all the SU and the TM sequences as far as the *Apa* I site in FeLV-A *env* (nucleotide number 1838; (Stewart *et al.,* 1986)) which code for the first 147 amino acids at the amino terminus. The fragments did not include the anchoring transmembrane region and were referred to as AHS, CHS, 215HS and VCHS respectively. They were all cloned directly into a preparation of *Bam* HI / *Sma* I digested pAcLacMCS which had been purified by agarose gel electrophoresis followed by electroelution. The recombinant plasmids, called pAcAHS, pAcCHS,

Figure 3.3 Oligonucleotide primers used for PCR



The chart shows the identity of the primers used during the course of the project. The numbers refer to the sequences of the strand of proviral DNA to which the primers are complementary and correspond to those used for pFGA by Stewart *et al.,* (1986). The arrow head denotes the 3' end of the primer and, by implication, the direction of extension. The primers which face downstream are complimentary to the antisense strand of the proviral DNA; those which face upstream are complimentary to the sense strand. MR 14 is a degenerate primer, containing 4 species in equimolar proportions. Shown below some of the primers are the nature of the non-complimentary sequences; the 'tail' refers to a GC rich clamp used to stabilise the PCR products.

Figure 3.4 Cloning strategy



Only certain relevant sites are shown for the following restriction enzymes; *Bam* HI (B), *Pst* I (P), *Sma* I (S) and *Bgl* II (Bg). The plasmids are presented in linear form and are not to scale. The p10 and polyhedrin (PH) promoters are shown together with their orientation. The FeLV proviral clones were contained in pUC derivatives.

pAcVCHS and pAc215HS respectively, were purified on a caesium chloride gradient.

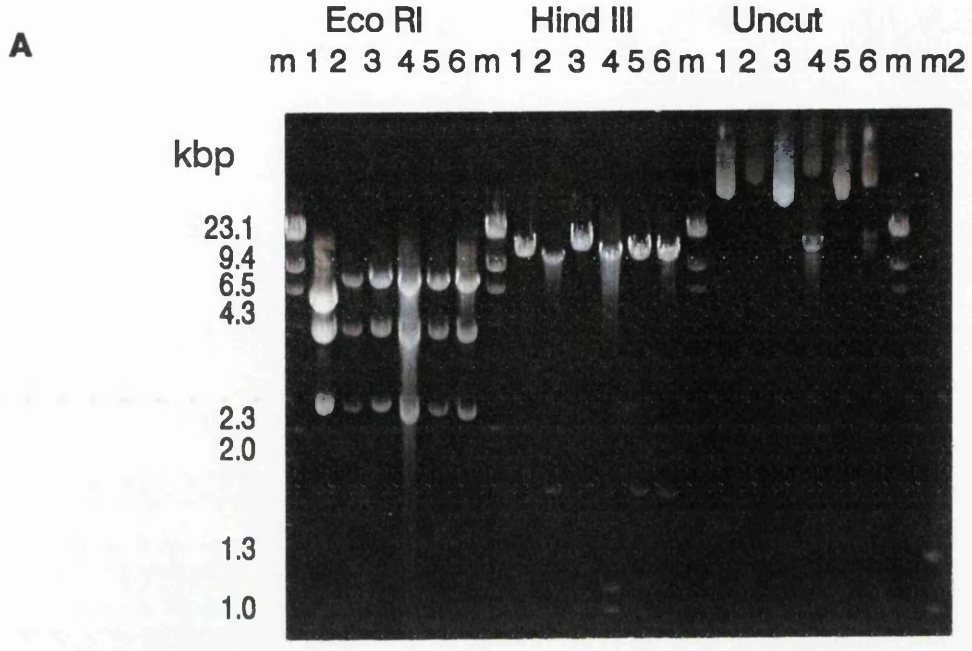
On attempting to repeat this cloning procedure using a pFGB template to produce the equivalent FeLV-B SU fragment, BHS, the PCR produced two species of product. One, as expected, was 1.8 kbp; the other was approximately 500 bp. The yields of the larger species could not be improved despite altering a number of parameters. Attempts at ligating the digested, gel purified product directly into pAcLacMCS failed. The fragment was, however, successfully subcloned into pBluescript. The resulting recombinant plasmid, pBMBHS, was analysed using multiple restriction enzyme digests, confirming that the insert was of the correct size and orientation. The BHS fragment was then subcloned into pAcLacMCS as a *Bam* Hl / *Sma* 1 fragment to generate pAcBHS.

To distinguish between the recombinant transfer vectors, each was digested with *Eco* Rl and *Hind* lll. The results of these digests are presented in Figure 3.5 (A). They show that all of the recombinant plasmids, but not the parent plasmid, have a 1.8 kbp insert between the two *Eco* Rl sites either side of the multiple cloning site. This result confirmed that the inserts were of the correct size. The *Hind* lll digests show that pAcLac-AHS, -VCHS and -215HS have a *Hind* lll site approximately 0.5 kbp from their 5' terminus. The vector containing the FeLV-C SU insert, pAcLac CHS, produced a different pattern of fragments confirming the existence of the Hind lll site near nucleotide 1100 (Riedel *et al.,* 1986) of the insert: pAcLacBHS has no Hind lll sites in the insert.

These results are entirely in accordance with predictions based on available sequence data. Results with *Acc* 1, *Cla* 1, *Apa* 1, *Bgl* ll and *Kpn* 1, were also in agreement with expectations (data not shown).

As the A/C subgroup phenotype is determined by a small region delineated by the *Hinc* ll and *Hind* lll sites (nucleotide numbers 276 and 532) (Rigby, 1989), the integrity of this region is vital to any experiments which investigate differences between these two subgroups. To demonstrate that the recombinant pAcLacMCS clones contained the same size of *Hind* lll / *Hinc* ll fragments as the proviral clones from which they were derived, a double digest was performed and the products were analysed on an 8% acrylamide gel (see Figure 3.5(B)). This experiment showed that a fragment of approximately 250 bp was absent from pAcLacCHS and pFSC and exhibited some size differences between the others. This fragment represents the 276-532 nucleotide region. The fragment from pAcAHS is slightly larger than that from pAcVCHS, which in turn is slightly larger than that from pAc215HS. The differences are also seen in the proviral clones. Analysis of

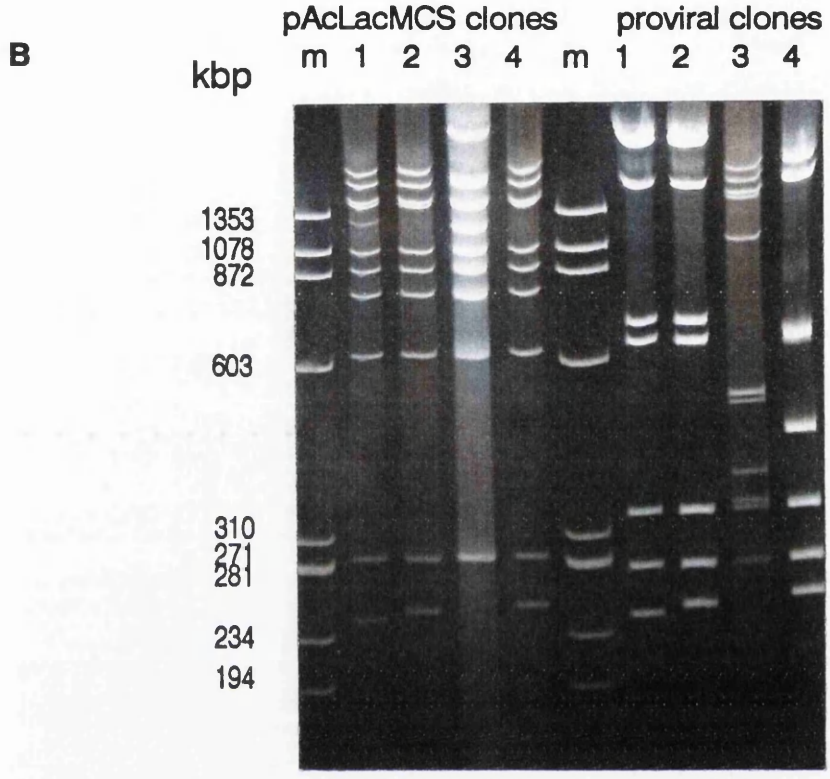
Figure 3.5 Restriction enzyme digests of plasmid clones



A. To distinguish between the 5 recombinant pAcLacMCS clones and the parent plasmid 2 μg of each plasmid was digested with 5 units of 2 restriction enzymes (*Eco* RI and *Hind* III) in the appropriate buffer. Undigested controls were also included. The products were separated on a 1% agarose gel using 40V overnight. The photograph shows the ethidium bromide stained gel.

Lanes: 1 = parent pAcLacMCS, 2 = pAcLacAHS, 3 = pAcLacBHS, 4 = pAcLacCHS, 5 = pAcLacVCHS, 6 = pAcLac215HS, m/m2 = molecular mass markers.

Figure 3.5(continued) Restriction enzyme digests of plasmid clones



B. To distinguish the pAcLacMCS recombinants containing FeLV-A, FeLV-C and the two A/C chimeras, a double digest with the restriction enzymes *Hinc* ll and *Hind* lll was performed on 5μg of plasmid. The products were separated on an 8% acrylamide gel using 50 V for 4 hours. Controls using the original proviral clones were performed in tandem. The photograph was then taken of the ethidium bromide stained gel.

Lanes: 1 = pAcLac215HS / pV215, 2 = pAcLacVCHS / pVC, 3 = pAcLacCHS / pFSC, 4 = pAcLacAHS / pFGA, m = molecular mass marker.

available sequence data suggested that the fragments from pAcLacVCHS and pAc215HS should be of the same size. This indicated that the original proviral clones might not be of the expected sequence.

DNA sequencing was performed using a commercial kit (Sequenase, Stratagene), as described in Chapter 2, to determine the sequences between the *Hind* lll and *Hinc* ll sites of the two chimeric proviruses. Each strand of DNA was sequenced separately. Sequencing revealed that the sequences were identical to the published sequences (Rigby, 1989; Rigby *et al.,* 1992). It was tentatively concluded that the apparent anomalies in fragment size observed on the polyacrylamide gel (Figure 3.5(B)) were due to the effects of secondary structure.

Using a mixture of multiple restriction enzyme digests and direct sequencing the recombinant pAcLacMCS vectors had been confirmed as containing the desired inserts in the correct orientation.

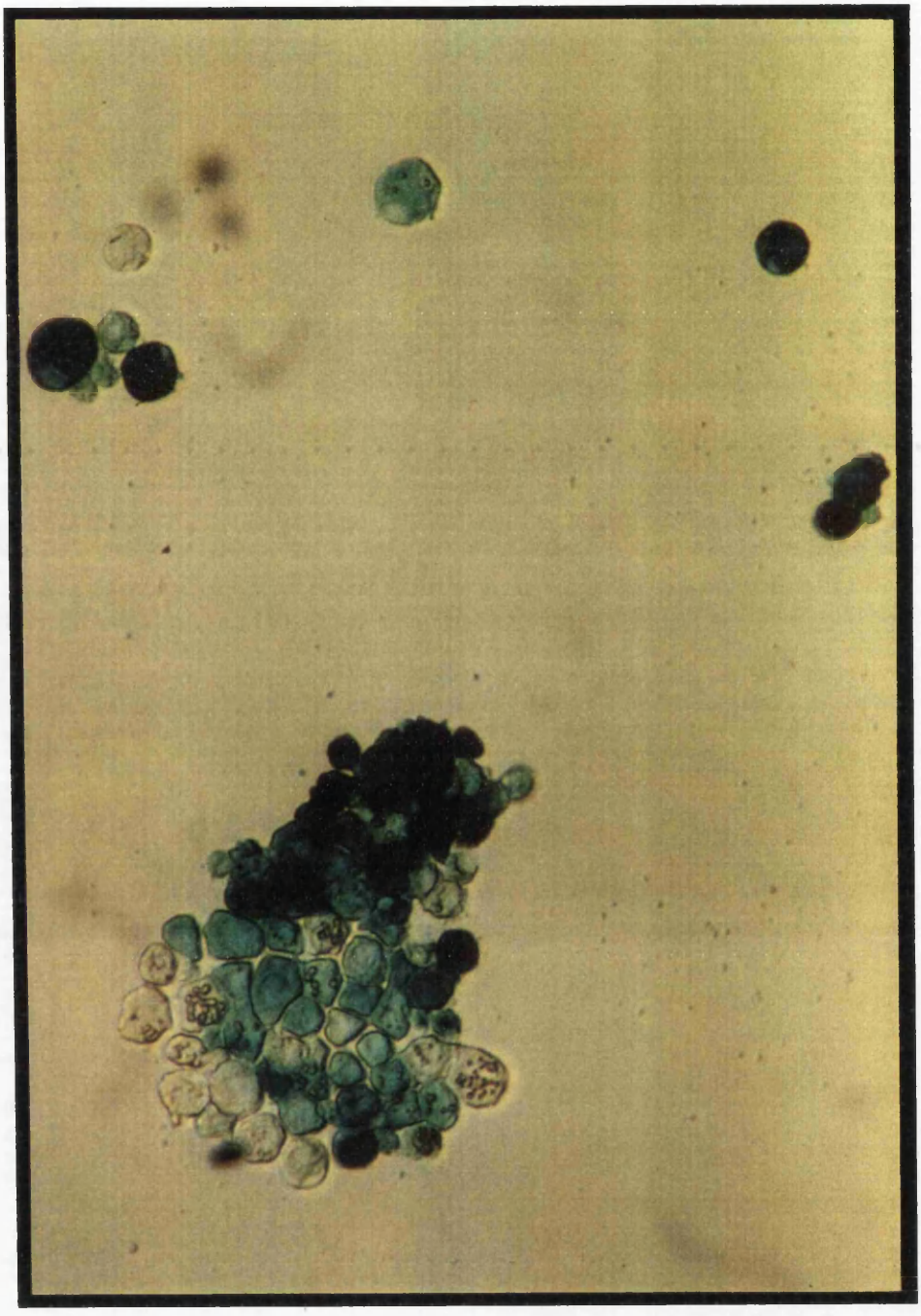
3.3.3 Production of Recombinant Viruses

The recombinant pAcLacMCS transfer vectors, pAcAHS, pAcBHS, pAcCHS, pAcVCHS and pAc215HS, were co-transfected with wild type baculovirus DNA into Sf9 insect cells. Following transfection the Sf9 cells were monitored twice daily for the presence of polyhedrin. Once numerous polyhedrin crystals could be seen, but before the majority of the cells had become infected, the supernatants were harvested by centrifugation at 3500 rpm for 10 minutes in a GPR bench centrifuge and then stored at 4^0C. The cells were fixed and then stained with X-Gal. On microscopic examination approximately 5% of the cells were positive for β galactosidase expression, indicating that recombinant viruses had been produced. The transfectant supernatants had average titres of approximately 10^4 pfu/ml.

The recombinant viruses were purified from the wild type viruses by a plaque purification technique (Brown and Faulkner, 1977) modified as described in Chapter 2. The β-galactosidase enzyme encoded in the recombinant viruses acted upon the colourless substrate X-Gal to produce a blue product allowing convenient selection of the recombinant viruses. An illustration of the stained cells is shown in Figure 3.6. Several plaques were picked from each round of purification. To achieve complete purification of the recombinant viruses 4 to 5 rounds of plaque purification were necessary.

During several stages of the purification procedure the stock of semi-purified recombinant virus was used to infect a 25 cm^2 tissue culture flask containing Sf9 cells at about 30% confluence. After infection the medium was removed and replaced with the serum free medium, SF900 ll. The supernatant was harvested 3

Figure 3.6 Sf9 cells infected with a mixture of recombinant and wild type viruses



The colour photograph shows Sf9 cells that were infected at sub-confluence with a mixture of recombinant (*lacZ* positive) and wild type viruses. The cells were left for 4 days post-infection before fixing with 2% formaldehyde and 0.2% glutaraldehyde and then staining with X-Gal. No agarose overlay was used in this experiment.
Some of the cells can be seen to contain small refractile crystals of polyherin, other cells are stained blue by the X-Gal. A few cells contain the polyhedrin crystals and are stained blue.

days later and concentrated using cellulose acetate miniconcentrators with a nominal molecular weight cut off of 30 kDa (Millipore). An aliquot of this concentrated supernatant was then used in a sandwich ELISA using the polyclonal anti-gp70 rabbit #709 serum and the anti-gp70 monoclonal antibody 3-17AP.

This ELISA was performed using the general method described in Chapter 2. The plate was washed with TBS-Tween between stages. A 1 in 1,000 dilution of the rabbit #709 serum in coupling buffer was used to coat the wells of a ELISA microtitre plate. The wells were incubated with blocking solution (3% Marvel in TBS) for a period of 2 hours and the antigens, diluted in 1% empigen in TBS, were then added. After a period of 3 hours, the wells were incubated with a 1 in 1,000 dilution of the alkaline phosphatase conjugated monoclonal antibody, 3-17AP, for a further hour. The assay was developed with 4-nitrophenyl phosphate in AP buffer.

The positive control used in this ELISA was a glutathione-S-transferase fusion protein (GST-E) which contained the oligopeptide previously defined as a neutralising epitope on gp70 (Elder *et al.,* 1987). The negative control used was a fusion protein (GST-G) which contained an oligopeptide derived from the VR 1 region (Rigby, 1989). These controls (obtained from Dr.N.Spibey) were used because native gp70 was found to be recognised relatively inefficiently in this assay. Control protein preparations derived from uninfected and wild type infected Sf9 cells were also included in this ELISA. These controls were designated C5 and C6 respectively.

Using this ELISA, recombinant FeLV SU was demonstrated in the supernatants from the pAcAHS, pAcCHS, pAcVCHS and pAc215HS / wild type DNA co-transfections. The positive control fusion protein, GST-E, reacted well: the negative control fusion protein, GST-G, did not react. No reaction was demonstrated with the control protein preparations C5 and C6 in this ELISA.

The supernatant from the pAcBHS / wild type AcMNPV co-transfection did not contain a detectable *env* product. Despite several attempts with a number of plaque purified isolates no recombinant protein could be demonstrated. No further analyses were undertaken.

The purified recombinant viruses which were freed of polyhedrin contamination were used to generate large volumes of high titre stocks for use in protein production work. Typically these stocks had titres in the range 2 x 10^7 to 8 x 10^7 pfu/ml. Concentrated stocks of virus were also made and then were used to prepare viral DNA for PCR purposes, as detailed in Chapter 2.

The recombinant viruses were termed AcAHS, AcCHS, AcVCHS and Ac215HS

respectively. The recombinant SU proteins produced were designated Bgp70-A, -C, -VC and -215 respectively.

3.3.4 Confirmatory PCR Reactions and Digests

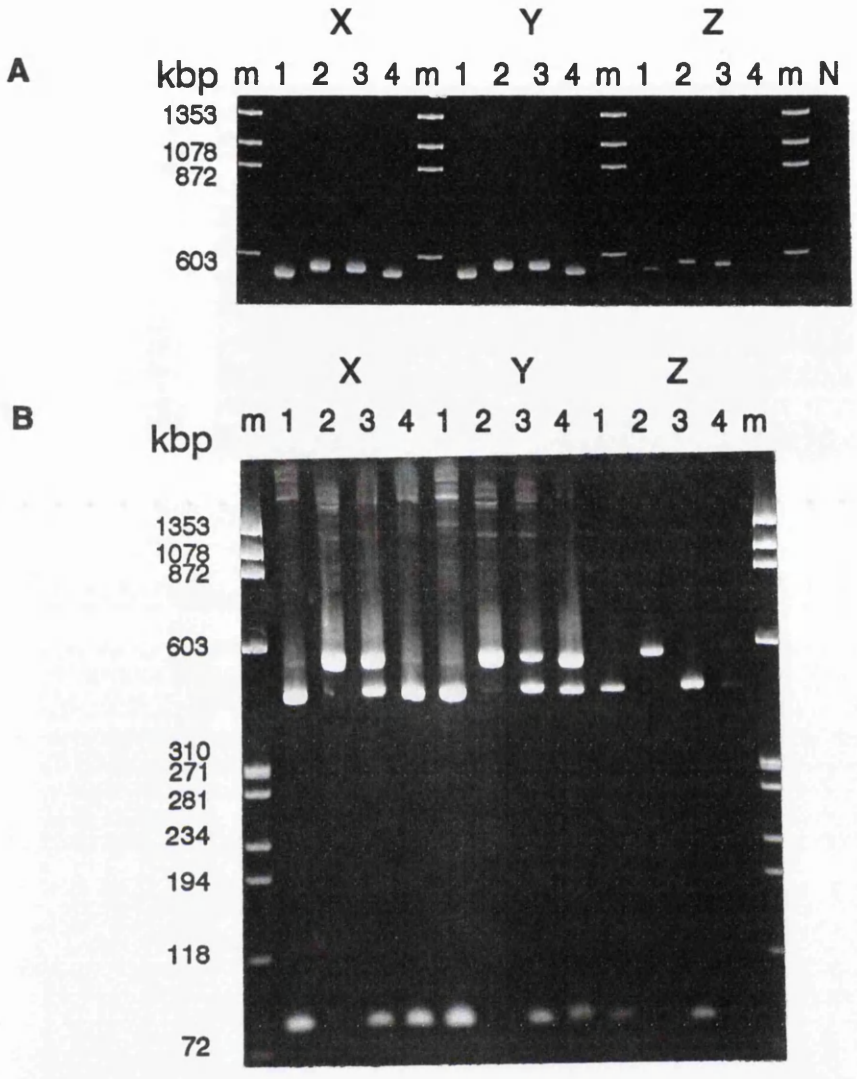
As mutations can occur during recombination and stocks of viruses can become contaminated it was decided to confirm that the recombinant viruses contained the correct FeLV coding sequences. This was achieved by initially generating fragments of FeLV VR1 from the recombinant baculoviruses by PCR. Several attempts were made to sequence the PCR products directly, without success. In the light of this failure, it was decided to use restriction enzyme digests of similar PCR products to partially confirm the identity of the recombinant viruses.

Two sets of PCRs were performed in tandem on each of the original proviral plasmids, the recombinant pAcLacMCS clones and the DNA obtained from the concentrated stocks of purified recombinant viruses. The reaction conditions for the PCRs were; annealing, 55°C, 1 minute; extending 72°C, 1 minute; denaturing, 90°C, 1 minute. The reactions were allowed to proceed for 30 cycles. Aliquots of 10 μl from each reaction were analysed by agarose gel electrophoresis. The remaining products were purified by phenol extraction followed by ethanol precipitation. Restriction enzyme digests were performed on the purified products. The first set of reactions used the oligonucleotide primers 18 and MR 14 (see Figure 3.3), followed by digestion with *Hind* lll. the second set used the oligonucleotide primers 7 and MR 14, followed by digestion with *Bgl* ll.

The results of the first set of reactions (18 + MR 14) are shown in Figure 3.7 (A). The PCR products showed some differences in size, the AcAHS product being noticeably smaller than the AcCHS product, contrary to expectations based on their predicted sequence. These differences were also seen in those reactions that used the proviral clones or the pAcLacMCS clones as templates. This result suggested that secondary sequence structure, which affected the results shown in Figure 3.5 (B), might also have influenced the outcome of this analysis. Digestion of the products of these reactions with *Hind* lll is shown in Figure 3.7 (B). Although incomplete digestion had occurred, it was clear that AcAHS, AcVCHS and Ac215HS had *Hind* lll sites whereas AcCHS had not.

The results of the second set of reactions are shown in figure 3.7 (C). Again size differences were observed which did not agree with expectations based on sequence data. In these reactions the fragments from AcAHS and AcCHS appeared to be of the same size, AcVCHS and Ac215HS being smaller. These differences were also seen in those reactions that used the proviral clones or the pAcLacMCS clones as templates. This suggested that secondary sequence structure might again be

Figure 3.7 Restriction enzyme digests of PCR products from recombinant viruses



A. 1 ng of template was added to a PCR reaction mix containing primers 18 and MR14 and the reaction allowed to proceed for 30 cycles under standard conditions. An aliquot of 10 µl of each 300 µl reaction was analysed by 8% PAGE using 100 V for 4 hours. The photograph is of the ethidium bromide stained gel.

B. Following the above PCR, the remaining products were phenol extracted, ethanol precipitated and then resuspended in the appropriate buffer. Each was then digested with 5 units of *Hind* lll. The products were separated as above.

Block X represents reactions that used the original proviral clones as templates, block Y the recombinant pAcLacMCS vectors and block Z the recombinant AcMNPVs.

Lanes: 1 = A (pFGA/ pAcLacAHS/AcAHS) 2 = C (pFSC etc.), 3 = VC (pVC etc) 4 = 215 (pV215 etc.) m = molecular mass markers, N = no template added.

influencing the result. Digestion of the products with *Bgl* II is shown in Figure 3.7 (D). The digest was incomplete. This gel demonstrated that the products derived from AcVCHS and AcCHS contained *Bgl* II sites. The products derived from AcAHS and Ac 215HS did not.

This pair of reaction sets demonstrated specific differences between the recombinant viruses, suggesting that they did contain different FeLV SU coding sequences. The variations in the restriction enzyme profiles were in accordance with expectations. This partially confirmed the identity of the recombinant viruses. To confirm fully that each VR 1 sequence had been preserved intact sequencing of the PCR products would be necessary. As direct sequencing failed, in the future it would be prudent to clone the products into plasmids and then sequence the products.

3.3.5 Production of Control Recombinant Baculovirus

It was decided that an additional control protein preparation would be required in cell binding studies which contained β galactosidase and an irrelevant recombinant retroviral SU protein. The availability of the FIV SU coding sequence of the UK8 isolate greatly assisted the generation of such a preparation.

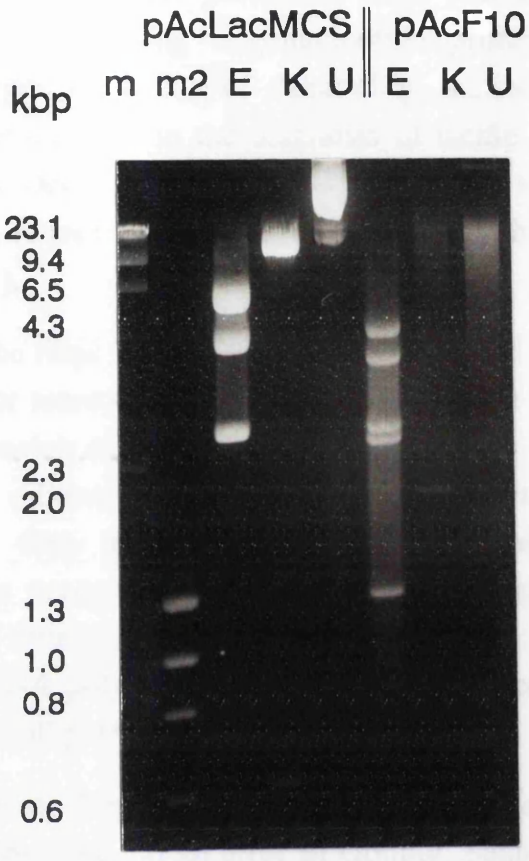
The SU coding sequence was subcloned from the plasmid p1-10 as a *Bam* HI / *Eco* RV fragment. This fragment was purified by agarose gel electrophoresis and inserted into a similarly purified *Bam* HI / *Sma* I digested preparation of the pAcLacMCS vector. The resulting recombinant plasmid, pAcF10, was analysed with a panel of restriction enzymes (see Figure 3.8). The plasmid pAcF10 contained a 1.3 kbp *Kpn* I fragment in accordance with predictions based upon sequence data.

The plasmid was then cotransfected with wild type AcMNPV DNA and a pure stock of the recombinant virus was prepared by plaque purification as described above. This stock was named AcF10. The ability of AcF10 to produce recombinant FIV SU was demonstrated by western blot as detailed in Chapter 4. The recombinant FIV SU was called Bgp100.

3.4 Discussion

This chapter describes the production by PCR of four recombinant baculoviruses containing fragments of FeLV *env* coding for the SU and part of the TM from FeLV-A, FeLV-C/Sarma and two A/C chimeric viruses, FeLV-A/C-Sarma and FeLV-A/C-FZ215 (Rigby, 1989). It also details the production of recombinant baculovirus which contained the coding sequence of the SU of the UK8 isolate of

Figure 3.8 Restriction enzyme digests of pAcF10



To distinguish between the FIV *env*-containing pAcLacMCS clone, pAc F10, and the parent plasmid 2 μg of each plasmid was digested with 5 units of 2 restriction enzymes (*Eco* Rl and *Kpn* l) in the appropriate buffer. Undigested controls were also included. The products were separated on a 1% agarose gel using 40V overnight. The photograph shows the ethidium bromide stained gel.

Lanes: E = *Eco* Rl cut plasmid, K = *Kpn* l cut plasmid, U = uncut plasmid, m/m2 = molecular mass markers.

FIV. All the recombinant viruses contained the *lacZ* gene under the control of the p10 promoter as a selectable marker.

PCR is a powerful method for generating large numbers of copies of a double stranded DNA template using oligonucleotide primers, a thermostable DNA polymerase and repetitive cycles of denaturing, annealing and extending. First developed as a method used in the diagnosis of sickle cell anaemia (Saiki *et al.*, 1985) it has since developed into one of the most widely used techniques in molecular biology. A recent review of current PCR technology has been published (Erlich *et al.*, 1991).

The results from the *Hinc* II / *Hind* II double digest had initially suggested that the sequences of one or more of the proviral clones did not agree with published data. Direct DNA sequencing of the chimeric proviral clones failed to identify any such inconsistency. A mixture of PCR, multiple restriction enzyme digests and sequencing were then used to confirm the identity of the recombinant baculoviruses. This process was thought to be necessary as an error could have been introduced at several stages in the cloning process. The lack of proof reading function by *Taq* DNA polymerase, mutation during recombination and laboratory contamination were all potential sources of such errors.

The failure to produce Bgp70-B despite the presence of β galactosidase positive plaques may have been due to an error in cloning. Sequencing of the vector could be performed to identify any defect in the region of the polyhedrin promoter. Alternatively, there may be some block in the production of FeLV-B SU in insect cells. As the insert had its own leader and start codon, an error introduced by the PCR remains the likeliest explanation. This result reinforces the care with which this technique should be routinely used in cloning reactions. Another attempt should be made to generate Bgp70-B. The availability of such a protein would allow the augmentation of the studies reported in later chapters.

No detailed PCR analysis was attempted on the control recombinant virus, AcF10, as the recombinant protein, Bgp100, expressed by it could be visualised on a western blot probed with the pooled infected cat serum P2 (data shown in Figure 4.3). The FeLV-A proteins did not react on similar western blots (data not shown). Such immunological techniques readily demonstrated that the control virus was distinct from the FeLV *env* containing viruses. The first description of the production of recombinant FIV SU using the baculovirus system has recently been published (Verschoor *et al.*, 1993).

The AcF10 virus encoded for the SU protein of the UK8 isolate of FIV but, because the 5' terminal 15 base pairs were derived from the Petaluma isolate, one amino

acid was altered (a glutamine instead of a glycine) from the predicted UK8 sequence. This amino acid change was in a conserved region of the protein (Rigby *et al.,* 1993) and was therefore unlikely to be of significance. This change was introduced by the necessity of designing PCR primers based on the original (i.e Petaluma) sequence when the molecular cloning of the UK8 isolate was being performed. This alteration was subsequently corrected and the intact FIV/UK8 sequence expressed in the baculovirus system by other workers in the Department of Veterinary Pathology (M.Rigby and A.Mackay, personal communication). There were no detectable differences in the immunological reactivity of the intact UK8 recombinant SU protein when compared with the slightly altered UK8 recombinant protein (data not shown).

The inclusion of the *lacZ* gene under the control of the p10 promoter allowed efficient selection of recombinant viruses. Plaque purification assays normally have to be left for 4-5 days before polyhedrin negative plaques can be seen (King and Possee, 1992). The use of the selectable marker reduced this time period to 2-3 days. The use of the *lacZ* gene with the p10 promoter in this manner has been reported by others (Vialard *et al.,* 1990). The gene can also be controlled by the *Drosophila melanogaster* heat shock promoter (hsp70) (Zuidema *et al.,* 1990).

During the course of this work the baculovirus expression system was developing rapidly. The use of linearised DNA, which is of relatively low infectivity, with a transfer vector that permits recircularisation, greatly increases the efficiency of recombination (Kitts *et al.,* 1990). This is because the transfer vector allows recircularisation of the linear DNA, introducing the desired gene. The number of wild type viruses recovered is therefore low. A further development uses linearised DNA from a β galactosidase positive baculovirus to provide a selectable marker for the identification of recircularised, non-recombinant viruses. This development reduces the number of plaque purification steps required (Harris and Coates, 1993). Such a system has the added advantage of only producing one recombinant protein. However, there is no evidence that the production of β galactosidase interferes with the production of other recombinant proteins. The major disadvantages with this technology are the potential complications that would result from cross-contamination of viruses produced using the two systems. In the above system β galactosidase positive baculoviruses are produced which do not contain the desired gene. In the pAcLacMCS system the β galactosidase positive baculoviruses do contain the desired gene. As such it was considered unwise to use the two systems simultaneously.

The very recent introduction of a method for the rapid production of recombinant baculovirus DNA in prokaryotic cells may also prove to be a valuable addition to

the system (Luckow *et al.*, 1993).

In conclusion, recombinant baculoviruses were produced which expressed truncated FeLV *env* genes from subgroups A and C. Baculoviruses which expressed the truncated *env* genes of two FeLV-A/C chimeras were also produced. The baculovirus system was found to be convenient and reliable to use. It was therefore decided to further investigate the production of recombinant surface glycoproteins in this system.

Chapter 4. Production of Recombinant Proteins

4.1 Introduction

This chapter describes the production, concentration and purification of one of the recombinant proteins, Bgp70-C, using the baculovirus AcCHS. Attempts to quantify the amount of recombinant protein produced are also discussed.

Having established a protocol for the production of Bgp70-C, it was possible to perform large scale preparations of each of the four recombinant FeLV SUs. The manufacture of three control protein preparations is also described.

4.2 Results

4.2.1 Initial Production of Recombinant Bgp70-C

Initially the production and concentration of recombinant protein using the AcCHS virus were studied. Once the production of the Bgp70-C protein had been optimised, two methods of purification were investigated.

(1) Optimisation of the production of recombinant Bgp70-C

Peak levels of polyhedrin production are usually observed about 54 hours post-infection. However, the optimum time to harvest recombinant proteins can vary from this figure (King and Possee, 1992).

An experiment was performed to determine both the optimum time to harvest the supernatant and the most efficient dose of virus to use. Six 25cm^2 flasks were seeded with 3 x 10^6 Sf9 cells. The next day the cells were inoculated with dilutions of the purified, titrated recombinant virus stock, AcCHS. After inoculation of the diluted virus, the flasks of cells were incubated for 2 hours on a tilting platform at room temperature. The inoculum was then removed and the cells were washed once in SF900 II serum free medium. The flasks of cells were incubated with 5 ml of fresh SF900 II medium for 6 days. At 28, 43, 56, 66, 76, 90, 103, 120 and 140 hours post-infection aliquots of 200 µl were removed and were then centrifuged briefly at 6,000 rpm in a benchtop centrifuge. The supernatants were removed and frozen. The quantity of protein produced was estimated using the rabbit #709 serum / monoclonal antibody 3-17AP sandwich ELISA (described previously in section 3.3.3). Each aliquot was diluted 1 in 2 with TBS / 1% empigen.

The controls used in this ELISA were the glutathione-S-transferase fusion proteins, GST-E and GST-G. GST-E contains the oligopeptide previously defined as a neutralising epitope on gp70 (Elder *et al.,* 1987). GST-G contains an oligopeptide previously defined as the VR 1 region (Rigby, 1989). The control protein

preparations, C5 and C6 (see section 3.3.3), were not included in this ELISA. The results of the assay are presented in Figure 4.1.

Altering the multiplicity of infection from 0.1 to 30 pfu/cell defined a range, 1-3 pfu/cell, which produced optimum protein levels. All subsequent infections were performed using viral inocula within the range.

Above this range, less protein was produced. At the higher multiplicities of infection (10 and 30 pfu/cell) the infected cells showed obvious changes in their morphology before 67 hours post infection. This result suggested that high doses of the virus were killing the cells before the polyhedrin promoter became fully active.

At the lowest multiplicity of infection (0.1 pfu/cell) it was observed that the cells became confluent before changes in their morphology were seen. It is known that baculoviruses replicate best in actively dividing cells (King and Possee, 1992).

It was found that the optimum length of time to incubate cells infected post-infection was between 67 and 76 hours. All subsequent infections were harvested after this time period.

If the cells were incubated for a longer period (6 days) then it was observed, using western blots, that the levels of recombinant protein rose within the cells. This may indicate that the active transport mechanisms in infected cells are preferentially affected before the protein manufacturing capability.

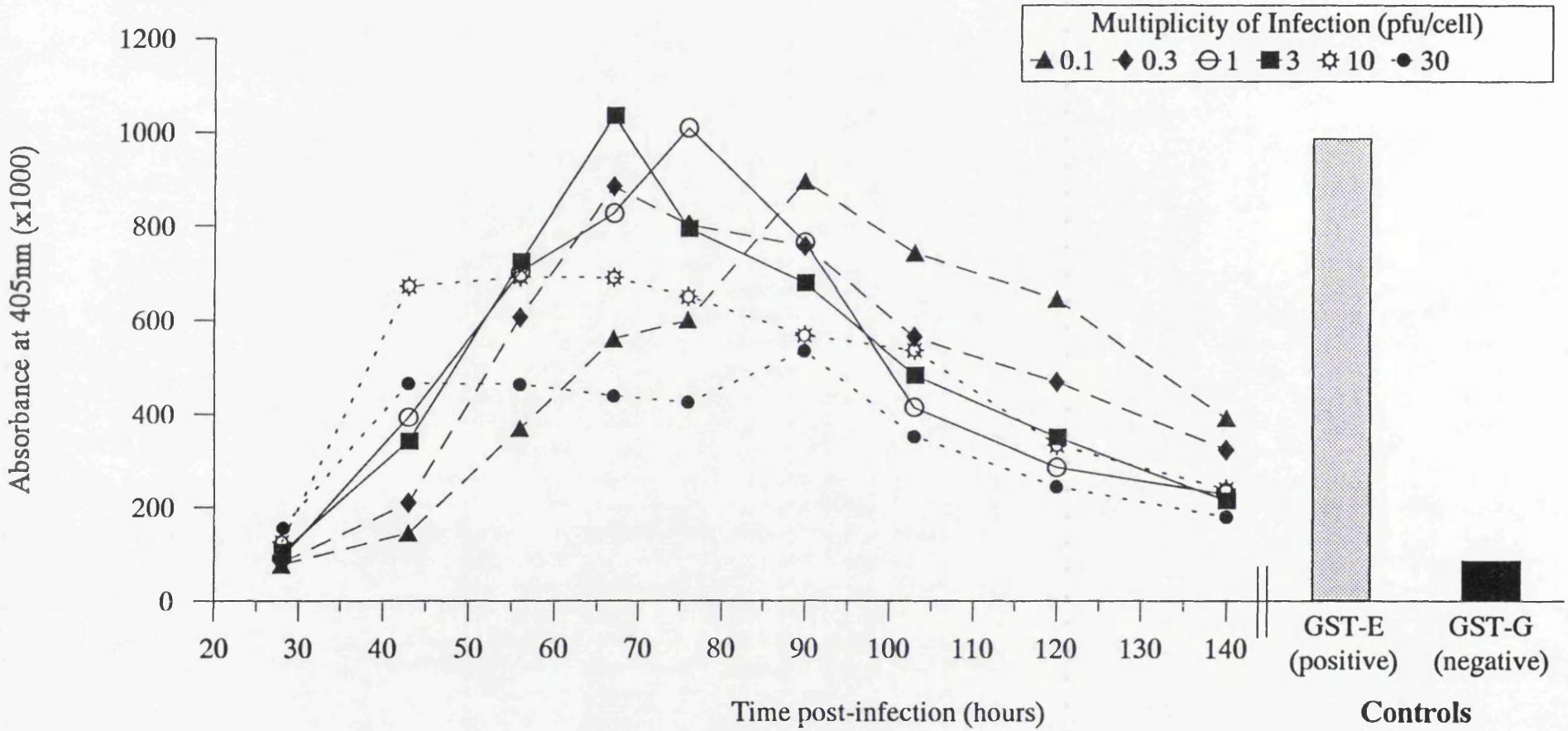
(2) Concentration of the recombinant protein

As the protein was secreted into the supernatant, concentration was considered essential before any purification steps were undertaken. In a series of experiments the most appropriate method of concentration of recombinant Bgp70-C was determined. For these experiments the recombinant protein was produced by infecting 25 cm^2 tissue culture flasks containing 3 x 10^6 Sf9 cells as described previously.

Ammonium sulphate precipitation (Harris, 1989) of the proteins was attempted, with little success. This might have been because the very low protein concentration in the supernatant prevented precipitation. Freeze drying was found to be effective, but suffered from the potential disadvantage of the coincidental concentration of salts from the SF900 II medium (Harris, 1989).

In contrast, the use of a Model 8200 stirred ultrafiltration cell (Amicon) proved an effective means of concentrating the supernatant with minimal increase in the salt concentration. Ultrafiltration was performed by first soaking a cellulose triacetate

Figure 4.1 Optimisation of protein production



To determine the optimum conditions for recombinant protein production, six 25cm^2 flasks were seeded with 3 x 10^6 Sf9 cells and were then infected with dilutions of the AcCHS virus. Aliquots of 200 µl were taken at intervals over a period of 6 days. The aliquots were clarified by centrifugation and the supernatants were frozen. The samples were then analysed using a rabbit #709 serum / monoclonal antibody 3-17AP sandwich ELISA as described in the text.

filter with a nominal molecular weight cut-off of 20 kDa (Sartorius) overnight in water. The next day the supernatant was harvested and was clarified by centrifugation at 5,000 rpm for 15 minutes in a JA14 rotor in a J2-21 centrifuge. The supernatant was then introduced into the assembled ultrafiltration cell and a pressure of 3.0 kg/cm^2 slowly applied using nitrogen. The ultrafiltration cell was checked at periodic intervals and refilled when the level of supernatant fell below 100 ml. When all of the supernatant had been filtered, the concentrate was diafiltrated with 400 ml of ice cold, sterile TBS. The supernatant was then finally concentrated to approximately 5% of its original volume.

The greatest disadvantage of this technique was the length of time required for concentration. Typically a flow rate of 1 ml per minute was achieved for the first 200 ml but, as the membrane pores became blocked, the flow rate steadily fell. Generally 400 ml of supernatants were ultrafiltered then diafiltrated in a single session which lasted 12 - 20 hours. The major determinant of the length of this period was the care with which all particulate matter was removed by centrifugation prior to ultrafiltration.

(3) Purification of the concentrated supernatants

Initially, purification of the concentrated supernatant was attempted using a nickel-nitrilotriacetic acid adsorbent immobilised on agarose (Hochuli *et al.,* 1987). The adsorbent was generously provided by Dr. G. Reid (Department of Veterinary Pathology, University of Glasgow). The supernatant and the adsorbent were incubated together for 1 hour at room temperature with gentle rotation. They were then separated by centrifugation at 2,000 rpm in a GPR bench centrifuge for 2 minutes. The adsorbent was washed with TBS until the OD_{280} of the washing was reduced to 0.04 or lower. The bound protein was eluted from the nickel-nitrilotriacetic acid adsorbent by incubation at room temperature with a solution of 100 mM EDTA in 100 mM Tris (pH = 8.0). The eluate and the adsorbent were separated after 1 hour incubation by centrifugation. The elution was repeated and the eluates were pooled. The eluates were dialysed overnight against TBS at 4^{0}C.

The purification of the protein was assessed by western blot using the monoclonal antibody 3-17AP. This was performed by incubation of a nylon membrane (prepared using the methods outlined in section 2.3.5) with a 1 in 600 dilution of 3-17AP in blocking solution. The results with the nickel-nitrilotriacetic acid adsorbent were disappointing in that only a fraction of the recombinant protein was recovered; the remainder did not appear to bind to the adsorbent. Despite investigating the effect of altering the pH of the binding phase, no improvement could be elicited. No further investigations into the failure of this system were

performed.

Experiments were then performed to investigate the efficiency of other methods in purifying the protein. In particular, lentil lectin affinity chromatography (Sutton, 1989) was performed by incubating the (20 x) concentrated supernatant with 1/10th of its volume of lentil lectin immobilised on Sepharose 4B (Pharmacia) for 2 hours at room temperature with gentle rotation. Centrifugation at 2,000 rpm for 2 minutes separated the lentil lectin from the supernatant. The lentil lectin was then washed with TBS until the OD_{280} of the washing was reduced to 0.04 or lower. The bound protein was eluted from the lentil lectin by incubation for 1 hour at room temperature with an equal volume of a solution of 0.5 M methyl-α mannopyranoside in TBS. The eluate and the lentil lectin were separated by centrifugation. The elution was repeated, and the eluates were pooled. The eluates were dialysed overnight against TBS at 4^0C.

The final volume of the eluate was half that of the concentrated supernatant. The overall concentration factor was therefore 40 fold.

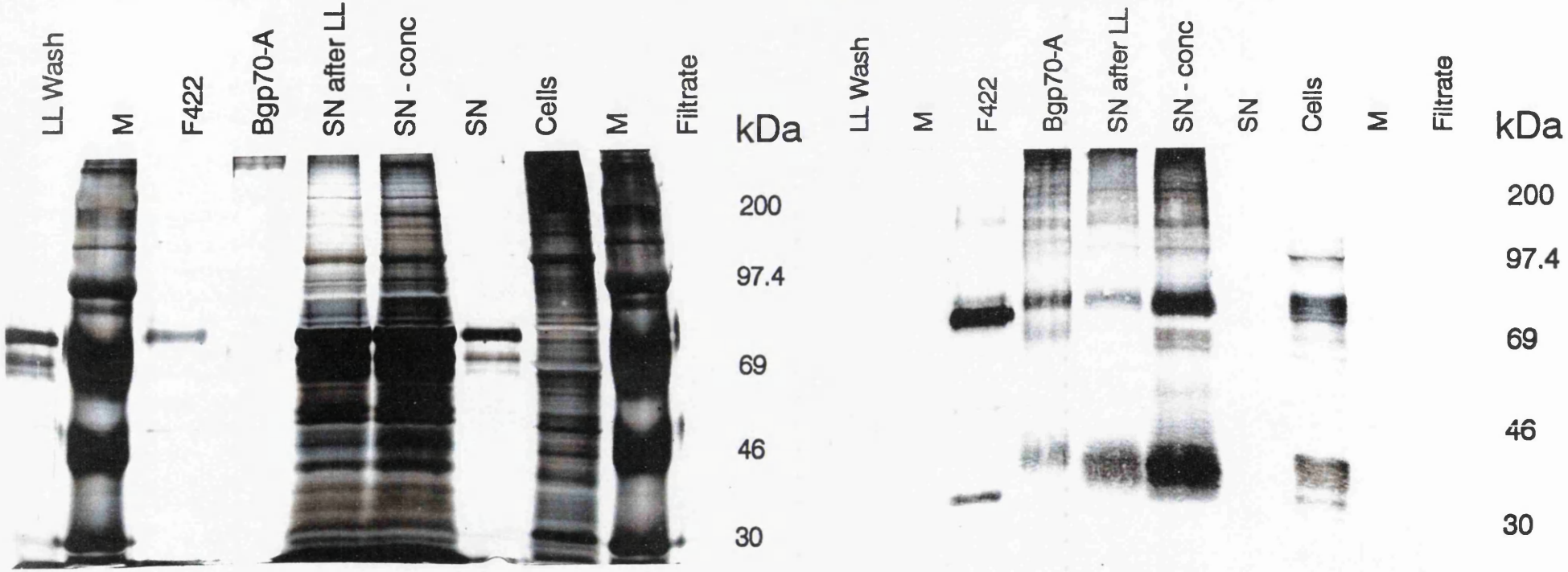
The various stages in the production, concentration and purification of the supernatant are shown in Figure 4.2. For the experiment shown in this figure the protein was prepared using 4 triple layered 225 cm^2 flasks with 2 x 10^7 cells per layer infected with 0.8 pfu/cell of the titrated purified stock of the FeLV-A SU containing baculovirus, AcAHS. After infection the cells were covered with a total volume of 380 ml SF900 II medium and were incubated for 72 hours. The Sf9 cells were harvested with the supernatant by tapping the flask to dislodge them. One flask was fixed and stained with X-Gal. Although most of the cells had become detached, those that remained were strongly β gal positive. The detached cells, which were precipitated by centrifugation, were resuspended in 10 ml of TBS and then 10 x lysis buffer (5 % NP40 in TBS) was added. The lysed cells were kept on ice for 5 minutes, before the lysate was cleared by centrifugation at 3,000 rpm for 10 minutes. The clarified supernatant was concentrated by ultrafiltration. The concentrated supernatant was then purified by lentil lectin affinity chromatography.

Aliquots of 30 μl were taken at various stages of this preparation and were analysed by electrophoresis through two 5-20% SDS-polyacrylamide gradient gels followed by either western blotting using the monoclonal antibody, 3-17AP, or silver staining. Western blotting and silver staining were performed by the general techniques outlined in Chapter 2. The western blot was performed by incubating the nylon membrane with 3-17AP, diluted 1 in 600 in blocking solution, for 2 hours at room temperature. After this time, the membrane was washed in PBS-Tween and then AP buffer. The blot was developed with NBT/BCIP in AP buffer.

Figure 4.2 The Production of Recombinant FeLV-A SU (Bgp70-A)

A) Silver stained gel

B) Western blot with monoclonal antibody 3-17AP



A 75 cm^2 flask containing 1 x 10^7 Sf9 cells was infected with 0.8 pfu/cell of the recombinant baculovirus, AcAHS. The cells were harvested 66 hours later then lysed with 0.5% NP40 (Cells above). The supernatant (SN) was concentrated 20 fold (SN-conc) by ultrafiltration, an aliquot of the filtrate was retained. The concentrated SN was then incubated with lentil lectin, washed with TBS (LL Wash) and eluted (Bgp70-A). Aliquots taken at various stages of the preparation were then separated by SDS-PAGE utilising two 5-20% gradient gels. One gel was then silver stained; the other was electroblotted onto a HyBond membrane and then probed with the monoclonal antibody 3-17AP. The membrane was stained with NBT and BCIP in alkaline phosphatase buffer. Also shown are a positve control derived from the FeLV producing cell line F422 and markers (M).

The silver stained gel illustrated in Figure 4.2 showed that the unconcentrated supernatant contained a single major protein species at 70 kDa as well as several minor species. No protein bands were seen in the filtrate suggesting that concentration of the supernatant by ultrafiltration was effective. The western blot (also Figure 4.2) demonstrated that there was a marked increase in the quantity of protein in the concentrated supernatant that reacted with 3-17AP. The silver stained gel also demonstrated that most of the proteins in the concentrated supernatant did not bind to lentil lectin. In contrast, the western blot showed a marked loss of protein in the supernatant, after lentil lectin affinity chromatography, that reacted with 3-17AP. Elution removed most of the recombinant protein from the beads and the eluate was seen on the western blot to be a single major protein band at 80 kDa with two lower bands, one faint and the other, at about 40 kDa, more intense. The eluate was seen on the silver stained gel to contain a single major species at about 70 kDa with a minor species at about 80 kDa. All the preparations also had small amounts of high molecular weight species. These may represent aberrantly glycosylated proteins or the poor solubility of the proteins in the gel. Poor gel solubility causes inconsistent migration of the proteins.

The cells were seen to have contained some protein which reacted with 3-17AP, but less than that present in the concentrated supernatant. This difference was ascribed to the relatively low multiplicity of infection and the length of time the preparation was incubated before harvest. There were no significant differences between the major products in the supernatant and the cells. All the recombinant protein preparations had large amounts of the 40 kDa species which reacted with 3-17AP. It is suggested that this protein represents a breakdown product.

The cells and supernatant both contained large quantities of β galactosidase. It can be seen as a 100 kDa band which reacted specifically with a monoclonal antibody on western blots (data not shown). The β galactosidase did not bind to the lentil lectin and could not be demonstrated in the eluate.

Also shown in Figure 4.2 is a preparation of native SU derived from lysed F422 supernatant as described in Chapter 2. This preparation was used as a positive control. It also has a breakdown product at about 35 kDa. This is common in preparations of native FeLV SU (Akerblom *et al.*, 1989).

(4) Quantification of recombinant Bgp70-A

The total protein in the eluate was measured as described in Chapter 2 and was found to be equivalent to 1 μg/10^6 Sf9 cells.

A mixture of 0.5 M methyl-αglucopyranoside together with 0.5 M methyl-

αmannopyranoside in TBS was used for the rest of the project in an attempt to improve the efficacy of elution from the lentil lectin. However, no quantitative assessment of any increase in protein content of the eluates resulting from this alteration was performed.

The optimised variables outlined above enabled as much as 4 μg of total protein to be produced from 10^6 Sf9 cells in later preparations.

Comparison between the silver stained gel and the western blot of the relative positions of the major bands in the F422 preparation and the eluate (Figure 4.2) indicated an anomaly. In the silver stained gel the major band in F422 was of a larger molecular mass than the major band in the preparation of Bgp70-A. In the western blot the major species in Bgp70-A was of a larger molecular mass than that in F422. This suggested that the major band in Bgp70-A was not that which reacted with 3-17AP in the western blot. It was concluded that the eluate was contaminated with a protein of slightly lower molecular weight than gp70.

4.2.2 Large Scale Preparations

To prepare large quantities of each protein for analysis the following procedure was used for each preparation. Ten 225 cm^2 flasks were seeded with Sf9 cells at a density of 2 x 10^7 cells per flask. The next day the cells were incubated for 2 hours with an inoculum containing 2 pfu/cell of one of the recombinant baculoviruses, AcAHS, AcCHS, AcVCHS, Ac215HS. The cells were then washed once with SF900 II serum free medium and were then maintained in SF900 II medium for 66 hours. The supernatant was harvested and then concentrated by ultrafiltration as described in section 4.2.1 b) above. Diafiltration was found to be unnecessary for lentil lectin binding (data not shown) and was not performed on these preparations. The concentrated supernatant was then purified by lentil lectin affinity chromatography. A mixture of 0.5 M methyl-αglucopyranoside together with 0.5 M methyl-αmannopyranoside in TBS was used to elute the recombinant protein. The resulting solution was then dialysed extensively against TBS at 4^0C. The proteins were stored in aliquots at -70^0C until used.

4.2.3 Production of Purified Control Protein Preparations

(1) FIV SU

To prepare large quantities of the recombinant FIV SU protein, Bgp100, ten 225 cm^2 flasks of Sf9 cells were prepared and infected as outlined above. The protein was purified by lentil lectin affinity chromatography from the concentrated supernatant as described before. The various stages in the production of

recombinant FIV SU are illustrated in Figure 4.3. The western blot illustrated that a large amount of the protein remains in the cell. This suggested that the recombinant FIV SU was not as well exported from the Sf9 cells as the FeLV SUs. The purification of the exported protein from the concentrated supernatant, using lentil lectin affinity chromatography, is also demonstrated.

A control preparation made from the FL4 cell line was used as a source of native FIV SU. The native protein can be seen as a heterogeneous band at approximately 110 kDa. The recombinant product was substantially smaller than the native protein. The apparent molecular mass of the recombinant protein was approximately 90 kDa.

(2) Uninfected control

To produce a control solution that contained only Sf9 insect cell proteins a large scale preparation was performed, as described above, but no virus was inoculated into the flasks. The supernatant was harvested and then concentrated to form a preparation that was called C5. This preparation was then purified by lentil lectin affinity chromatography and the resulting control preparation was designated C5L.

The total quantity of protein in C5L was larger than that observed with virus-infected cells. This was thought to be due to the maintenance of the rapid growth rate of the cells in the absence of virus.

(3) Wild type infected control

To produce a control solution that contained baculoviral proteins a large scale preparation was performed as described above. The cells were infected with wild type AcMNPV (2 pfu/cell) and then were incubated for 66 hours. The supernatant was concentrated to form a preparation called C5, which was then purified using lentil lectin affinity chromatography. The purified preparation was designated C6L.

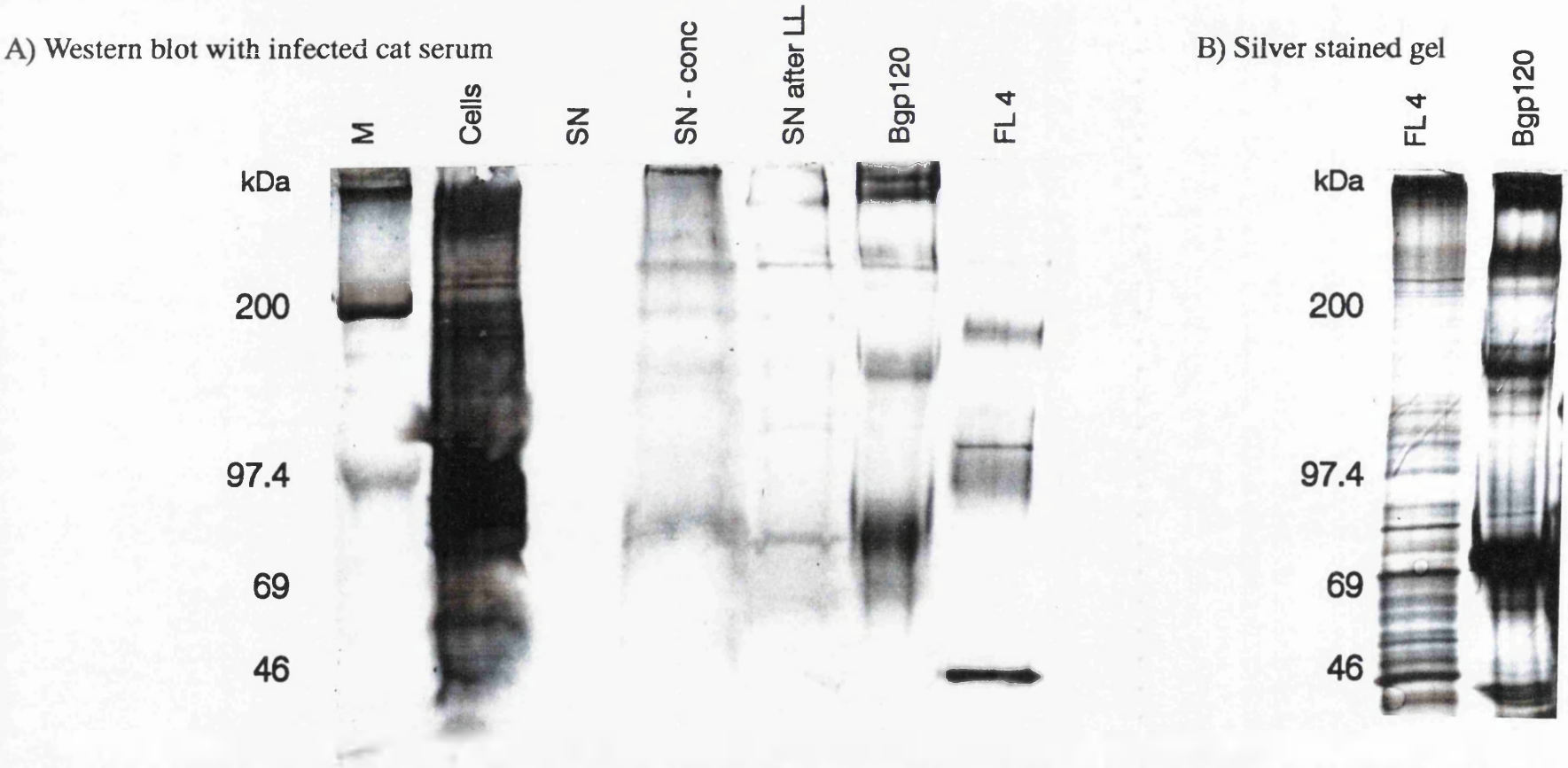
The total quantity of protein in this preparation was slightly less than the average protein concentration of the recombinant FeLV SU preparations.

4.3 Discussion

This chapter details the analysis of the production of one recombinant protein, Bgp70-A, which was then purified from concentrated supernatant by lentil lectin affinity chromatography. It also details the large scale manufacture of 4 recombinant FeLV SU proteins (Bgp70s) and three control protein preparations (Bgp100, C5L and C6L).

The eluates of Bgp100 and the Bgp70s all contained at least one major impurity, a

Figure 4.3 The Production of Recombinant FIV SU (Bgp120)



A 75 cm^2 flask containing 1 x 10^7 Sf9 cells was infected with 2 pfu/cell of the recombinant baculovirus, AcF10. The cells were harvested 66 hours later then lysed with 0.5% NP40 (Cells above). The supernatant (SN) was concentrated 20 fold (SN-conc) by ultrafiltration and then was purified using lentil lectin affinity chromatography (Bgp120). Aliquots taken at various stages of the preparation were then separated by SDS-PAGE on two 7.5% gradient gels. One gel was then silver stained; the other was electroblotted onto a nitrocellulose membrane. The membrane was probed with the pooled infected cat serum, P2, and then with a goat anti-cat IgG-alkaline phophatase conjugate (SeraLab). The membrane was stained with BCIP/NBT in AP buffer. Also shown is a positive control derived from the FIV producing cell line FL4 and molecular mass markers (M).

species of 60-69 kDa. This protein was probably the major baculoviral envelope glycoprotein, gp64 (Volkman, 1986). As this protein was glycosylated it would be expected to co-purify with the recombinant protein when using lentil lectin affinity chromatography.

The lack of binding of the recombinant Bgp70s to the nickel-nitrilotriacetic acid adsorbent may have been due to several factors. The histidine residues may not have been exposed on the surface of the recombinant protein. Alternatively, post-translational modification or non-specific proteolysis could have removed them. Finally, the charges on the histidine residues could have been masked because the isoelectric points of glycoproteins are generally lower than their non-glycosylated counterparts. This difference is due to the acidic nature of the carbohydrate side chains.

Lectins are a large family of ubiquitous carbohydrate binding proteins that are widely used in molecular biology for the purification of glycoproteins (Sutton, 1989). As lentil lectin is known to bind mannose residues well, and insect cell glycosylation produces mannose-rich carbohydrate side-chains (Kuroda *et al.*, 1990), no investigation was undertaken using other lectins. A more detailed discussion of insect cell glycosylation is presented in Chapter 5.

The major product which reacted with 3-17AP in the eluate was slightly larger than the SU from F422. The difference in size was probably partly due to the presence of this non-anchoring portion of TM. This could be confirmed with a monoclonal antibody specific for the amino portion of TM. The calculated relative molecular mass of this portion was 15 kDa (and of the whole TM protein, 22 kDa). The predicted relative molecular mass of the recombinant protein was therefore 85 kDa. However, the observed relative molecular mass was approximately 80 kDa. The difference was probably due to aberrant glycosylation of the recombinant protein. The difference between the size of the recombinant FIV SU product, Bgp100, and the native FIV SU was probably also due to the aberrant glycosylation of the insect cells. The glycosylation of the recombinant proteins was further investigated using enzymatic digestion with endoglycosidase (see section 5.2.4).

It is not possible to state a definitive figure for the quantity of recombinant proteins in the preparations. However, it is clear that relatively little protein was produced compared with the reported values of some baculovirus expressed proteins. Early experiments had relied on Coomassie stained gels of the concentrated impure products to estimate quantity. However, the preparations contained several other proteins that interfered with this form of analysis. In particular, species at 70 kDa were a cause of some initial misplaced optimism. The nature of these species was

never formally determined, but it is likely that they were serum albumin and, to a lesser extent, the baculovirus p74 protein (Rohrmann, 1992).

In attempting to express membrane proteins in the baculovirus system, other workers have also found that yields were disappointingly low (King and Possee, 1992). This may appear to be a limitation of the baculovirus system which will have to be considered in the future when selecting a system for recombinant protein production. However, the baculovirus expression system does produce some proteins well. Up to 50% of the total cellular protein can be of recombinant origin (Matsuura *et al.,* 1987). The proteins that are expressed well such as β galactosidase (Pennock *et al.,* 1984), β interferon (Smith *et al.,* 1983b) and influenza HA (Kuroda *et al.,* 1986), are often not, or only sparsely, glycosylated. The high level of expression of β galactosidase by insect cells was confirmed in these experiments. Approximately 30% of the relative molecular mass of FeLV SU is composed of carbohydrate. In the case of the FIV SU this figure rises to 50%. It is possible that the degree of glycosylation required by the protein influences the ability of the insect cell to produce that protein. It is more relevant to compare the expression of FeLV SU with other, heavily glycosylated, retroviral SU proteins that have been expressed using the baculovirus vector system.

There have been several reports of the expression of entire retroviral *env* genes in insect cells (Wells and Compans, 1990; Thomsen *et al.,* 1992; Arp *et al.,* 1993; Verschoor *et al.,* 1993). These reports indicate that the proteins are retained within the cells, sometimes in inclusion bodies (Arp *et al.,* 1993). Such inclusion bodies assist subsequent purification, though potentially denaturing concentrations of urea or guanidinium hydrochloride are required for solubilisation. Thomsen *et al.* (1992) expressed FeLV *gag* as well as the entire *env* and found that the immature particles produced by the *gag* containing recombinant virus would incorporate the *env* gene product when an *env* containing recombinant virus was used as well. Apart from the use of these immature particles, no attempt was made to isolate the *env* protein. It would be interesting to discover if the *env*-containing baculovirus did produce inclusion bodies. The inclusion bodies may increase protein stability thereby increasing the yield. The authors also note that their product was not processed into gp70 and p15(E). There was no evidence of such cleavage in the recombinant proteins described in this thesis. Some investigators report cleavage of HIV *env* (Wells and Compans, 1990), while others do not (Rusche *et al.,* 1987; Hu *et al.,* 1987). The HTLV-1 and FIV *env* products are not cleaved (Arp *et al.,* 1993; Verschoor *et al.,* 1993).

There have also been several reports of the expression of truncated *env* in insect cells. Noteborn *et al.* (1990), expressed only the SU component of ALV in insect

cells but, unexpectedly, the protein was not transported into the supernatant. They suggest that the lack of TM was responsible. The TM of RSV is known to be necessary for the stabilisation of an external glycoprotein oligomer which is required for efficient extracellular transport (Einfeld and Hunter, 1988). This may explain the apparent lack of expression in the supernatant of the preliminary experiments using the *Bam* Hl / *Sst* ll segment of FeLV *env*. This segment only contained 15 amino acids of TM. In contrast the successful *Bam* Hl / *Apa* 1 fragments contain 147 amino acids. This would suggest that the components of TM required for oligomerisation reside in this 142 amino acid residue region. The advantage of producing a secreted product is that it greatly facilitates subsequent purification. The relative lack of export of the recombinant FIV SU may have been due to a similar lack of oligomerisation. The production of a recombinant baculovirus containing a truncated FIV *env* lacking the transmembrane domain of the anchoring TM protein would enable further analysis of the transport mechanisms in insect cells. Such a recombinant protein might also be more efficiently exported from the cells, facilitating purification and increasing the yields of protein obtained. The relevance of such studies to the transport in higher eukaryotic cells has not been ascertained.

One report of HIV *env* expression (Wells and Compans, 1990) is of particular interest as it describes the production of a recombinant baculovirus that expressed a truncated version of HIV *env* which lacked a transmembrane domain and was therefore analogous to the work presented here. These authors found that the protein was mainly secreted into the supernatant. They also demonstrated that the recombinant protein specifically bound to CD4, even in the absence of proteolytic cleavage. This encouraged studies, reported in Chapter 6, which examined the interaction of the recombinant protein with cell surface proteins.

The quantification of recombinant proteins requires some care. The use of immunoreactivity as a measure of recombinant protein (Hu *et al.,* 1987) is highly dependent upon the assay used. As recombinant baculoviral proteins are aberrantly glycosylated the immunoreactivity may differ markedly from the native protein. This is particularly true when a polyclonal serum is used since the great majority of antibodies may be directed against the carbohydrate components of the retroviral envelope (Elder *et al.,* 1986). This point will be illustrated further in Chapter 5. The level of expression quoted by these authors (which assuming 6 x 10^8 cells/litre of culture fluid was equivalent to 2-3 $\mu g/10^6$ cells) could not be confirmed independently as neither of the other two reports of HIV *env* expression in insect cells (Rusche *et al.,* 1987; Wells and Compans, 1990) quote a figure. Noteborn *et al.* (1990) quote a figure for the production of ALV SU of 1-2 $\mu g/10^6$ Sf9 cells.

They do not, however, state the method which they used to arrive at this figure. As their product had an apparent molecular mass of 65 kDa (due to incomplete glycosylation) and was not purified from cell lysates, contamination of the 65 kDa species with the baculoviral envelope protein gp64 must be considered a possibility. Arp *et al.* (1993) report a recombinant HTLV-1 *env* product that is equivalent to 10 $\mu g/10^6$ cells. This result was obtained from analysis of the purified inclusion bodies. However the majority of the protein produced was not glycosylated. The component that was glycosylated did not contain a large amount of carbohydrate. This may, in part, explain the larger quantities produced. FeLV, FIV, ALV and HIV all have much greater quantities of carbohydrate in their SU proteins.

The detailed molecular factors determining the level of expression of the polyhedrin gene, or its equivalent following recombination, are not well characterised. The 5' non-coding region of the polyhedrin gene is known to be required for high levels of expression. The seven 5' nucleotides, TATAAATA, in this region are of particular importance (Matsuura *et al.,* 1987). The polyhedrin promoter is known to be generally more efficient than the p10 promoter (Min and Bishop, 1991). It is also known that polyhedrin production is not directly proportional to viral replication and that all genes that have been expressed at a high level have been derived from cDNA or genomic clones that do not contain introns (Luckow and Summers, 1988). The lack of detailed information on factors which govern the expression of foreign genes is a common disadvantage with eukaryotic protein expression systems. In common with other eukaryotic systems, the unpredictable nature of recombinant protein production is a major disadvantage of the baculovirus expression system. Even in recombinant baculoviruses containing the same foreign gene the level of expression seems to vary (Luckow and Summers, 1988). Several isolates were made of each recombinant baculovirus reported here, though no detailed analysis of the levels of protein production was undertaken.

To produce larger quantities of protein would require the development of a specific protocol for this purpose. The use of spinner cultures has been advocated (King and Possee, 1992). However attempts to adapt Sf9 cells to serum free medium spinner cultures failed. As Sf9 cells can tolerate low (2%) serum concentrations (data not shown), it may be possible to use small amounts of serum to avoid these adaption problems. Concentration of 10% serum-containing medium by ultrafiltration results in rapid membrane fouling. The use of spinner cultures is probably best restricted to the production of proteins that are retained within the Sf9 cells. A similar problem would probably exist with the air-lift fermentation vats that are used for the industrial scale production of recombinant baculovirus proteins (Luckow and

Summers, 1988).

In conclusion, few reports of the expression of retroviral SU proteins record a reliable figure for the quantity of protein produced. This may suggest that the truncated FeLV SU/TM recombinant proteins described in this thesis may not be produced in unusually low quantities for this system. Similarly, the quantities of FIV SU cannot be determined, even in relation to the quantity of FeLV SU. It is suggested that the degree of glycosylation required by the protein may influence the total yield obtained. Further analysis, possibly using mutated SUs with fewer glycosylation sites, is required.

Chapter 5. Analysis of Recombinant Proteins

5.1 Introduction

This chapter describes detailed analyses of the four recombinant FeLV SU proteins. These experiments characterised the interaction of anti-gp70 antibodies, particularly virus neutralising antibodies, with the proteins. The experiments also examined whether the proteins' expected subgroup phenotypes were identifiable by immunological methods.

Western blotting was performed to examine the ability of the antibodies to bind linearised polypeptides. Enzyme linked immunoabsorbent assays (ELISAs) were then performed to investigate the ability of the antibodies to bind to the antibody-captured proteins. ELISAs are thought to be more representative of the antigen/antibody interaction *in vivo*. Finally the ability of the recombinant proteins to block the action of virus neutralising antibodies was investigated. These assays were used to examine the ability of the recombinant proteins to function in a similar manner to the native SU glycoproteins. The value of any recombinant protein is largely determined by its ability to mimic its wild type counterpart. Recombinant proteins expressed in the baculoviral system are sometimes immunologically distinct from their native counterparts (Luckow and Summers, 1988).

5.2 Results

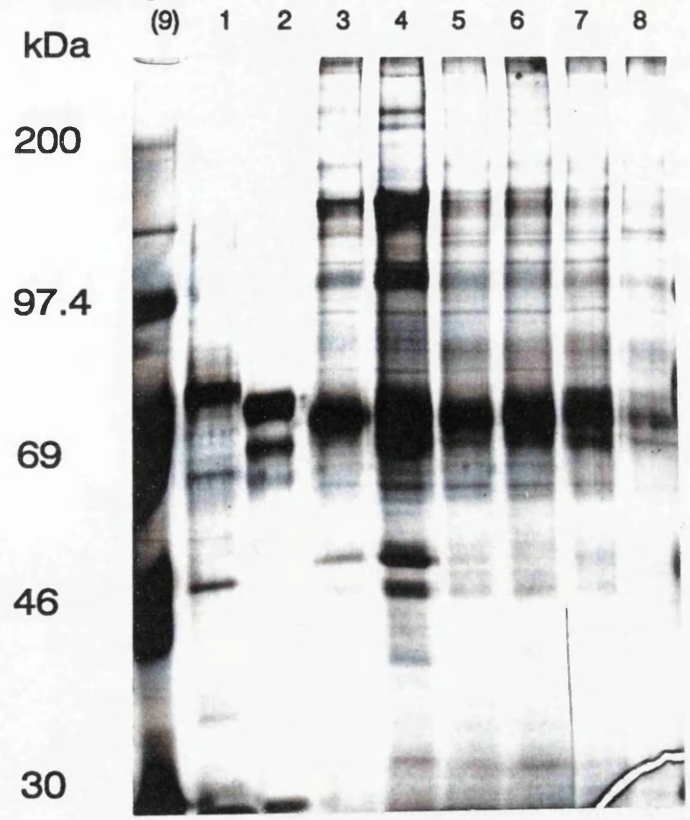
5.2.1 Western Blotting

Western blots were performed to analyse the binding of five sera to the four recombinant proteins, Bgp70-A, -C, -VC and -215. The blots were performed as outlined in Chapter 2. The membranes were washed between stages for one hour with PBS-Tween. The proteins were separated by 7.5% SDS-PAGE before electroblotting onto nitrocellulose membranes. The negative control preparations, C5L and C6L, were included in these analyses (see 2.3.2 and 2.3.3). Two positive control preparations of FeLV purified from F422 and FL74 cells were also included. The antibody preparations used were 3-17AP, rabbit #709 serum, rabbit #395 serum, cat P11 serum and cat 'Wendy' serum, which are defined in Chapter 2. A silver stained gel was also prepared of the same proteins. The results of these analyses are presented in Figure 5.1

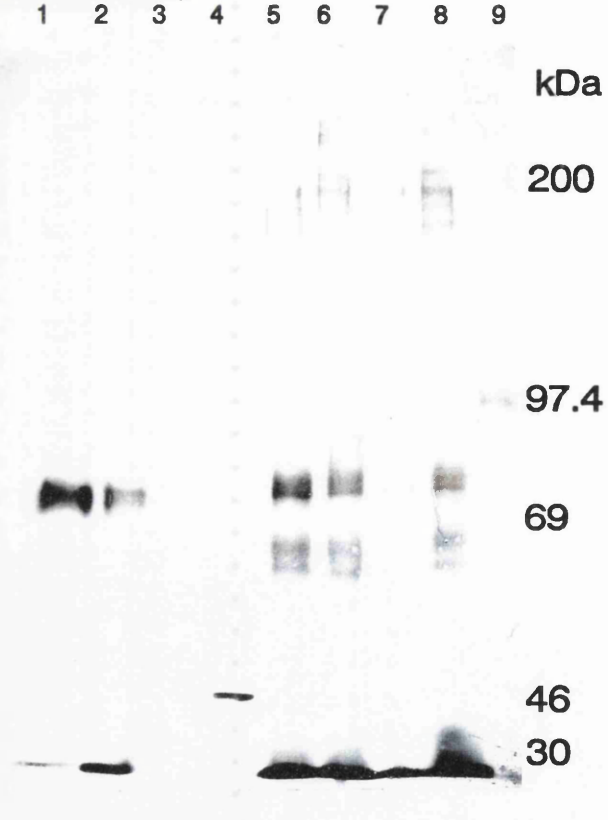
To demonstrate the binding of the monoclonal antibody 3-17AP to the four Bgp70s, the antibody was diluted 1 in 600 with blocking solution and was then incubated with one of the nitrocellulose membranes for 2 hours. The membrane was developed with a solution of NBT and BCIP in alkaline phosphatase buffer.

Figure 5.1 Analysis of recombinant FeLV SU's

A) Silver stained gel



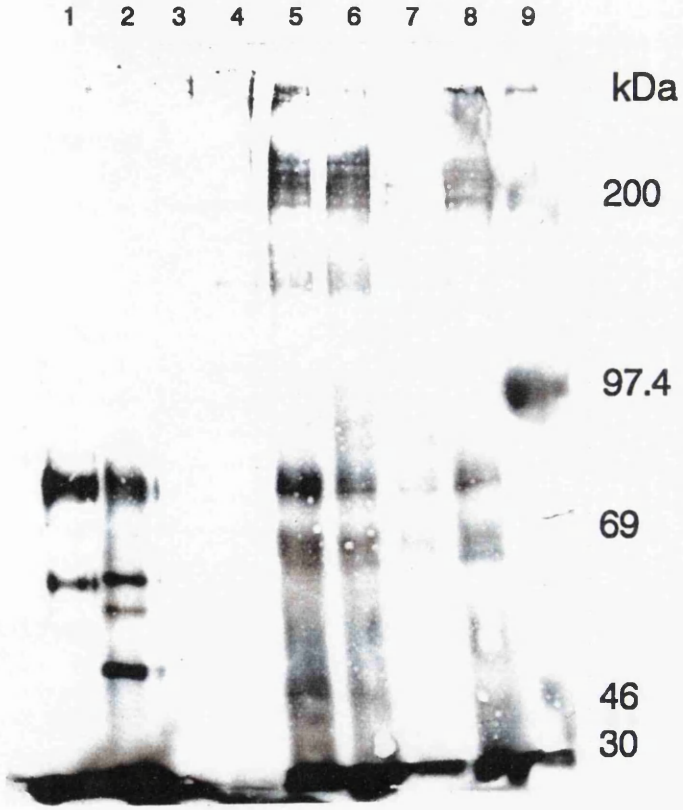
B) Monoclonal antibody 3-17AP



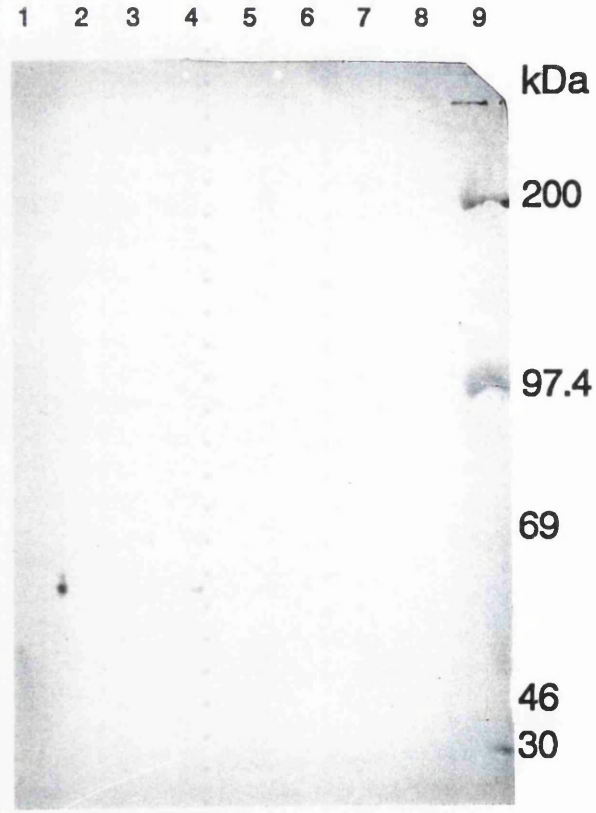
To visualise the 4 recombinant proteins and the various controls used in this figure a 7.5% SDS PAGE gel was run and silver stained. Similar gels were used throughout this figure for the western blots. To demonstrate that an alkaline-phosphatase linked, anti-gp70 monoclonal antibody, 3-17AP, specifically bound to the recombinant FeLV SU's a nitrocellulose membrane was probed with a 1 in 600 dilution of 3.17AP. Control protein preparations of wild type infected and uninfected supernatants were included (C6l and C5L respectively), as well as two FeLV positive controls.
Lane 1, FL74; Lane 2, F422; Lane 3, Wild type infected; Lane 4,Uninfected;Lanes 5, 6, 7, 8, Recombinant FeLV SU's Bgp70 -215, -VC, -C, -A respectively; Lane 9 molecular mass markers

Figure 5.1 Analysis of recombinant FeLV SU's (continued)

C) Rabbit serum #709



D) Rabbit serum #395

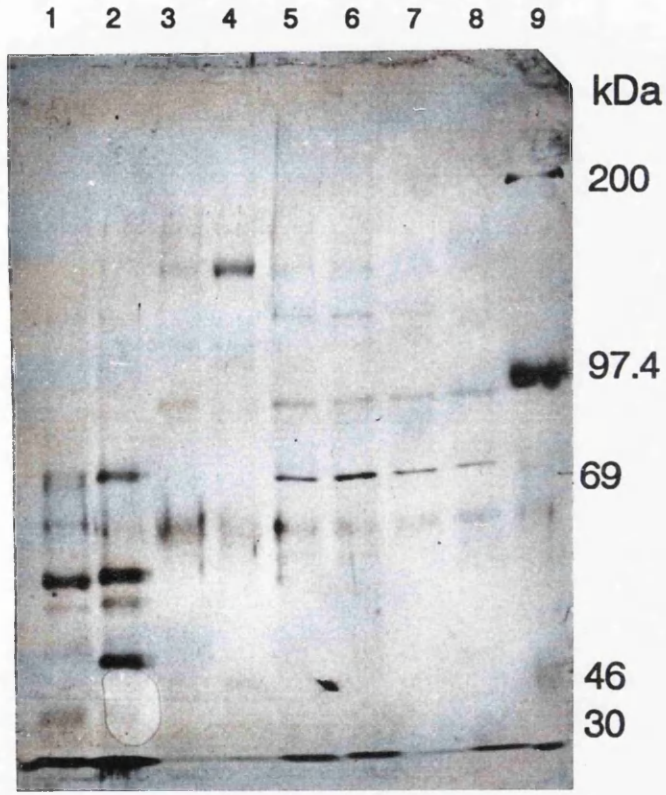


To demonstrate that the rabbit #709 anti-FeLV rabbit serum specifically bound to the recombinant FeLV SU's, the four proteins were analysed by western blot. The nitrocellulose membrane was probed with a 1in 500 dilution of #709 and then was incubated with agoat anti-rabbit IgG - alkaline phosphatase conjugate (BioRad). Control protein preparations of wild type infected and uninfected supernatants were included, as well as two FeLV positive controls. An identical membrane was similarly prepared and probed with the SPF rabbit serum, #395.

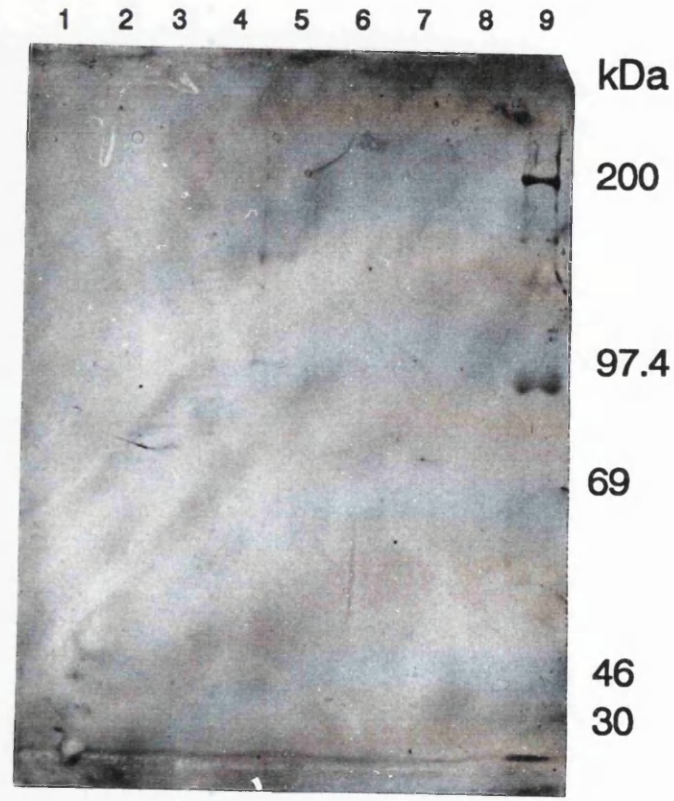
Lane 1, FL74; Lane 2, F422; Lane 3, Wild type infected; Lane 4,Uninfected;Lanes 5, 6, 7, 8, Recombinant FeLV SU's Bgp70 -215, -VC, -C, -A respectively; Lane 9 molecular mass markers

Figure 5.1 Analysis of recombinant FeLV SU's (continued)

E) Cat serum P11



F) Cat serum Wendy



To demonstrate that the recovered cat serum, P11, specifically bound to the recombinant FeLV SU's, the four proteins were analysed by western blot. The nitrocellulose membrane was probed with a 1 in 100 dilution of P11, washed, and then incubated with a goat anti-cat IgG - alkaline phosphatase conjugate (SeraLab). Control protein preparations of wild type infected and uninfected supernatants were included, as well as two FeLV positive controls. An identical membrane was similarly prepared and probed with the SPF cat serum, "Wendy".
Lane 1, FL74; Lane 2, F422; Lane 3, Wild type infected; Lane 4,Uninfected;Lanes 5, 6, 7, 8, Recombinant FeLV SU's Bgp70 -215, -VC, -C, -A respectively; Lane 9 molecular mass markers

To investigate the binding of the rabbit #709 anti-gp70 serum to the recombinant proteins, another membrane was incubated for 2 hours with #709 serum, diluted 1 in 300 with blocking solution. Following this step, a goat anti-rabbit IgG - alkaline phosphatase conjugate (BioRad), diluted 1 in 1,000 with blocking solution, was incubated with the membrane for 1 hour. The membrane was developed as above. A similar blot was prepared using the SPF rabbit #395 serum.

To investigate the binding of recovered cat P11 serum to the recombinant proteins a fourth membrane was incubated for 2 hours with the serum diluted 1 in 100 with blocking solution. Following this, a goat anti-cat IgG - alkaline phosphatase conjugate (SeraLab), diluted 1 in 500 with blocking solution, was incubated with the membrane for 1 hour. The membrane was developed as above. A similar blot was performed using the SPF cat 'Wendy' serum.

The pattern of binding to the recombinant proteins observed with the rabbit anti-gp70 serum was similar to that observed with the monoclonal antibody 3-17AP. Both the rabbit #709 serum and the 3-17AP antibody bound to a lower 65 kDa species as well as the major 80 kDa species. This result suggested that the epitope recognised predominantly by the hyperimmune serum may have been the same as that recognised by 3-17AP. In contrast, the pattern of binding seen with the recovered cat serum was different. The P11 serum did not bind to the lower 65 kDa species and bound only to a component of the broad 80 kDa band. This finding suggested that P11 serum may have predominantly recognised different epitopes. It was not obvious why P11 did not bind to the 65 kDa species, as it probably represented the small amount of SU portion that was cleaved from the TM during production. It is possible that the quantity of protein present was insufficient to provide a visible reaction.

Both #709 and P11 sera bound to a 55 kDa species in FL74 and F422 derived virions and a to 50 kDa species in F422 derived virions. These species probably represent breakdown products of gp70. As neither were recognised by 3-17AP, it is suggested that the portion containing this epitope had been cleaved off. This result also demonstrated that #709 serum did have antibodies to epitopes in native FeLV SU, other than that recognised by 3-17 AP. The importance of this observation becomes apparent when considering the testing of developmental FeLV vaccines in species other than the cat. If such animals predominantly respond with neutralising antibodies to one epitope, which the cat seems unresponsive to, then the value of such experiments is reduced.

It was interesting to compare the relative reactivities of the gp70 derived from F422 and FL74. The 3-17AP antibody and the #709 serum appeared to bind to more of

the FL74, suggesting that more FL74 derived gp70 was present. However, the recovered cat P11 serum bound to more of the F422 gp70. The reason for this difference was probably due to the relative abundance of FeLV-A in the two preparations. P11 serum was derived from a cat that had recovered from an infection with FeLV-A and therefore may mainly recognise this subgroup. Further investigations into this observation are presented below.

The silver stained gel showed that the major bands in the two FeLV preparations were of different sizes, yet all three of the anti-gp70 antibody preparations recognised components in the two preparations that were of the same size. This suggested that the major band in the F422 preparation, previously assumed to be gp70, might be another protein. A likely candidate is bovine serum albumin, which is approximately 70 kDa in size.

The binding of 3-17AP to a 46 kDa protein in the uninfected control preparation, C5L, could not be readily explained. Similarly, the binding of #709 to a band at 150 kDa was probably a spurious reaction to an insect cell component. The SPF rabbit #395 serum did not show such binding.

5.2.2 ELISAs

To investigate further the interaction of the recombinant proteins with various sera a number of ELISAs were performed. The techniques used are outlined in Chapter 2. The ELISA microtitre plates were washed 4 times between each stage with TBS / 0.05% Tween 20.

(1) Rabbit #709 serum / MAb 3-17AP ELISA

To demonstrate that the anti-gp70 monoclonal antibody 3-17AP bound to the recombinant proteins a sandwich ELISA was performed. This ELISA was also used during the purification of the recombinant baculoviruses and the optimisation of protein production (see sections 3.3.3 and 4.2.1).

The controls used in this ELISA were the glutathione-S-transferase fusion proteins GST-E and GST-G. GST-E contains the oligopeptide previously defined as a neutralising epitope on gp70 (Elder *et al.,* 1987). GST-G contains an oligopeptide derived from the VR 1 region. The control preparations, C5L and C6L, were also included in this ELISA (see section 4.2.3). The rabbit #709 anti-gp70 serum, diluted 1 in 1,000 with coupling buffer, was incubated overnight (100 µl per well). The next day the plate was blocked by incubating with 2% Marvel in TBS (200 µl per well). Threefold dilutions of the antigens in 1% empigen in TBS were then incubated with the plate for 3 hours (100 µl per well). The initial quantities of

protein were 10 μg (GST-G and -E), 4 μg (C5L, C6L, Bgp70-A and -215) or 1.3 μg (Bgp70-C and -VC). The plate was then incubated with a 1 in 1,000 dilution of 3-17AP in 2% Marvel in TBS (100 μl per well) for 1 hour. The reaction was developed by adding 50 μl of a 1 mg/ml solution of 4-nitrophenyl phosphate in AP buffer to each well and incubating for approximately 4 hours. The results of this ELISA are presented in Figure 5.2.

The three negative control preparations, (GST-G, C5L and C6L) did not react in this experiment. In contrast the positive control, GST-E, reacted well, confirming the specificity of the assay. The four recombinant proteins also reacted well. These reactions were greater than the F422 derived gp70 (data not shown).

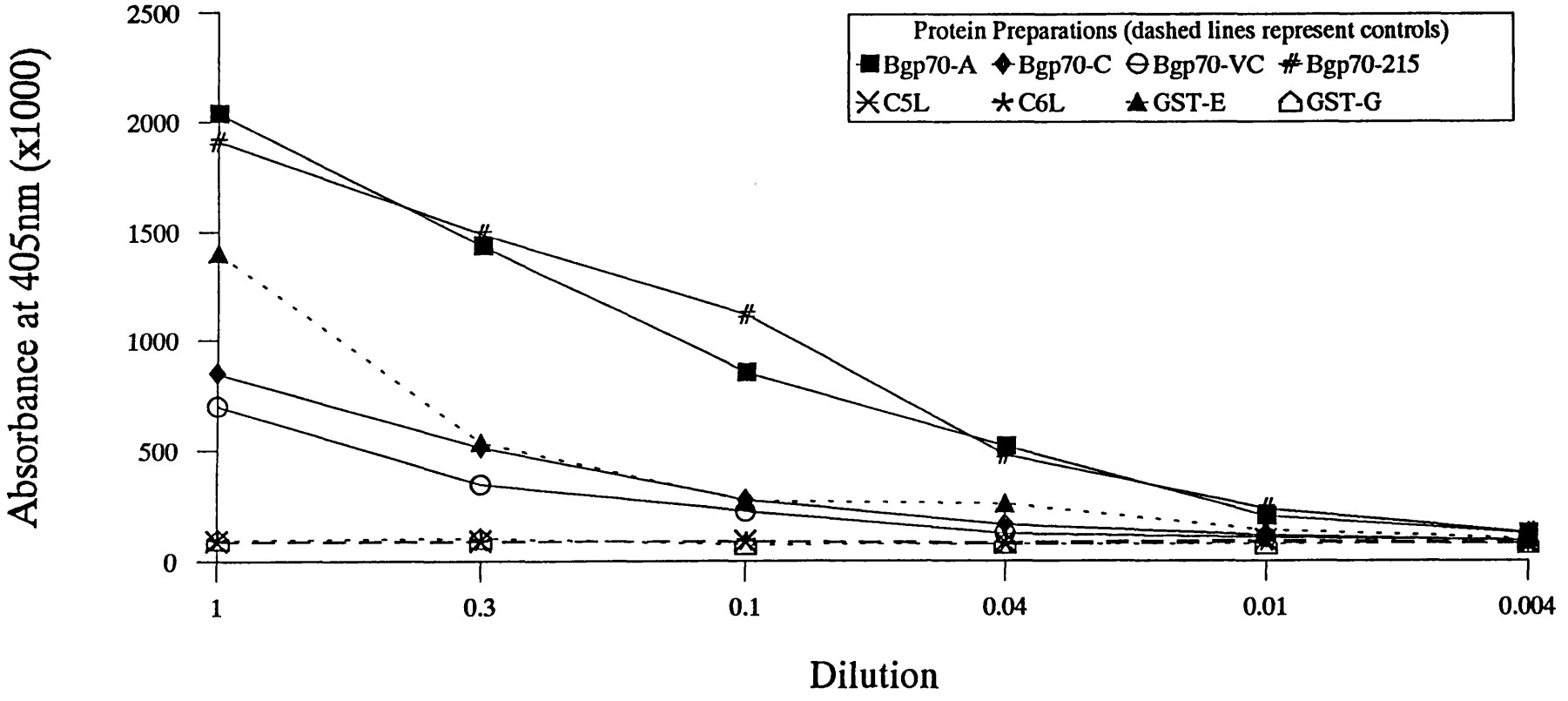
(2) MAb 3-17 / cat serum ELISA

A set of ELISAs were performed to investigate the ability of two recovered cat sera, P11 and Cat 31, to bind to the recombinant proteins. The ability of Cat 31 serum, which is FeLV-C/Sarma specific, and P12, which is FeLV-A specific, to discriminate between the various recombinant proteins was also analysed.

The monoclonal antibody 3-17 was obtained as ascites from Dr. K. Weijer. This preparation was not alkaline phosphatase linked. It was coupled to an ELISA plate by diluting 1 in 1,000 with coupling buffer and incubated in a 96-well plate overnight (100 μl per well). The next day the plate was blocked with 2% Marvel and 10% goat serum in TBS (200 μl per well). Five-fold dilutions of the antigens in 1% empigen in TBS were then incubated with the plate for 3 hours (100 μl per well). The quantities of the recombinant proteins used were 4 μg (C5L, C6L, Bgp70-A and -215) or 1.3 μg (Bgp70-C and -VC). The plate was then incubated with a 1 in 200 dilution of serum for 2 hours followed by a 1 in 600 dilution of a goat anti-cat IgG - alkaline phosphate conjugate. 2% Marvel in TBS (100 μl per well) for 1 hour. The reaction was developed by adding 50 μl of a 1 mg/ml solution of 4-nitrophenyl phosphate in AP buffer to each well and incubating for approximately 4 hours. Equal volumes of C5L and C6L were mixed together and used as a negative control. FL74 derived gp70 was used as a source of FeLV-C/Sarma and FeLV-A gp70, and F422 derived gp70 as a source of FeLV-A gp70 alone. The results of this experiment are presented in Figure 5.3.

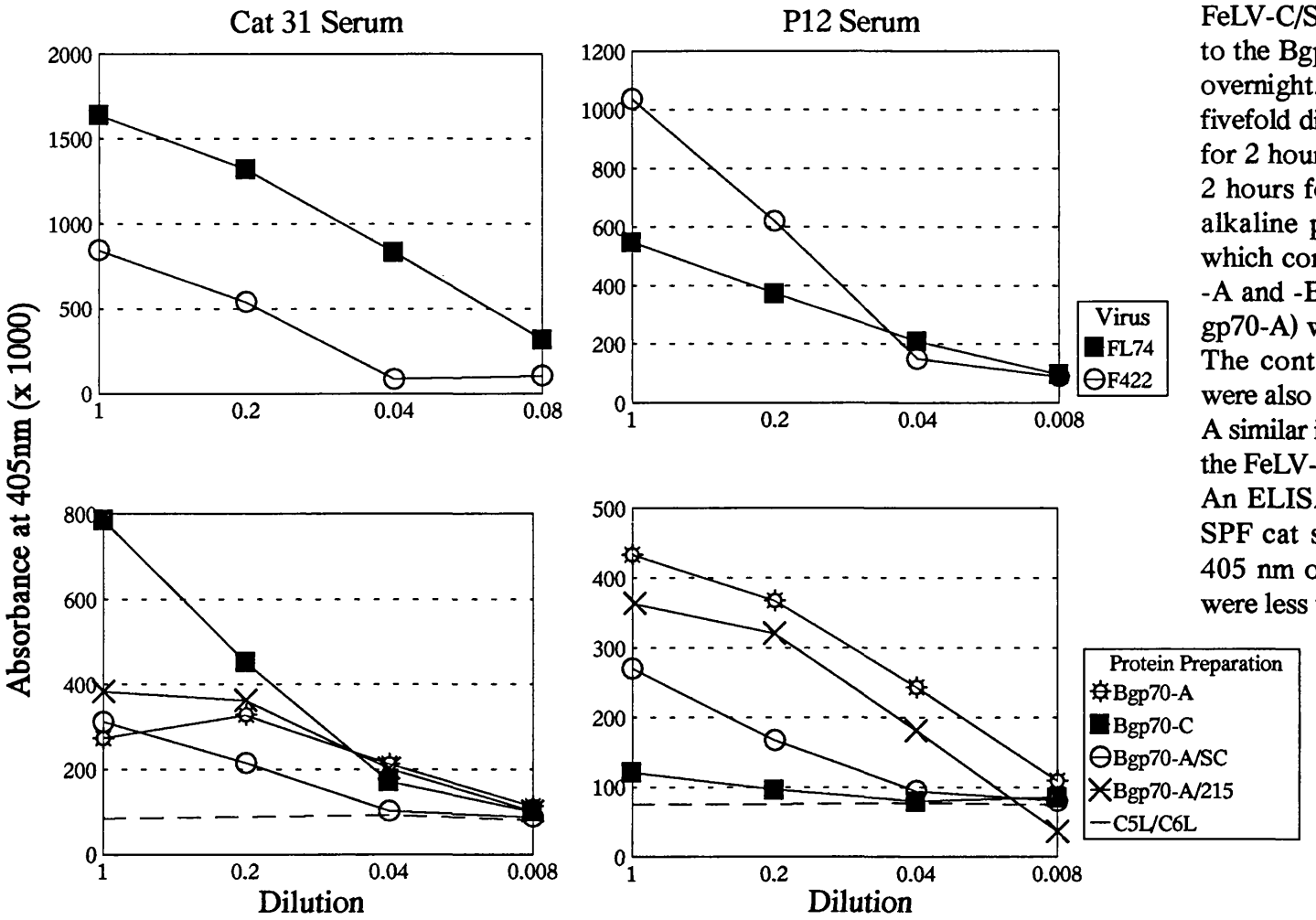
The reaction of Cat 31 serum with FL74 was much greater than with F422. In contrast, the reaction of P12 with F422 was greater than with FL74 (reflecting the relative abundance of FeLV-A gp70 in the two preparations). This partially confirms the reported subgroup specificities of these sera (O.Jarrett, personal communication). To confirm these observations fully would require isolated FeLV-C/Sarma gp70.

Figure 5.2 Rabbit serum #709 / monoclonal antibody 3-17AP sandwich ELISA



To demonstrate the detection of the Bgp70 proteins using the rabbit anti-gp70 serum, #709, and the MAb 3-17AP an ELISA was performed. The #709 rabbit serum (diluted 1/1000 with coupling buffer) was incubated overnight on a ELISA microtitre plate. The next day the plate was blocked, then threefold dilutions of the Bgp70's were added for 2 hours. The recombinant proteins were detected by incubation of the plate with the MAb 3-17AP followed by the addition of the substrate 4-nitrophenylphosphate. The glutathione-S-transferase fusion proteins (GST-E and GST-G) were included as positive and negative controls respectively. The similarly purified preparations of uninfected (C5L) and wild type (C6L) supernatants were also included.

Figure 5.3 Monoclonal antibody 3-17 capture ELISA



To investigate the ability of the FeLV-C/Sarma specific serum, C31, to bind to the Bgp70's, 3.17 ascites was incubated overnight. The plate was then blocked and fivefold dilutions of the proteins incubated for 2 hours. The test serum was added for 2 hours followed by a goat anti-cat IgG-alkaline phosphatase conjugate. FL74 - which contains native gp70-C (as well as -A and -B) and F422 (which contains just gp70-A) were included as positive controls. The control preparations C5L and C6L were also included.

A similar investigation was undertaken with the FeLV-A specific serum, P12.

An ELISA was also performed with the SPF cat serum, Q25. The absorbance at 405 nm of all the samples in this ELISA were less than 0.080(data not shown).

The reaction of Cat 31 serum was much greater with Bgp70-C than with the other three Bgp70s. The reaction of P12 with Bgp70-C was much less than with the other Bgp70s. This suggested that the majority of the antibodies in both sera were directed against regions other than that which determines subgroup specificity.

(3) Subgroup-specific MAb ELISA

A third ELISA was performed with the subgroup specific monoclonal antibodies C1G10, HL2 and HL5. These antibodies are all neutralising in vitro, but differ in their titres against the three FeLV subgroups (see Table 2.1). Two monoclonal antibodies were used as controls in this experiment; 6-15, which is similar to the broadly neutralising 3-17 monoclonal antibody, and VPG 68, which can function as a capture antibody for native FIV SU in ELISAs. Only three of the Bgp70 preparations (Bgp70-A, -C and -215) were used in this experiment.

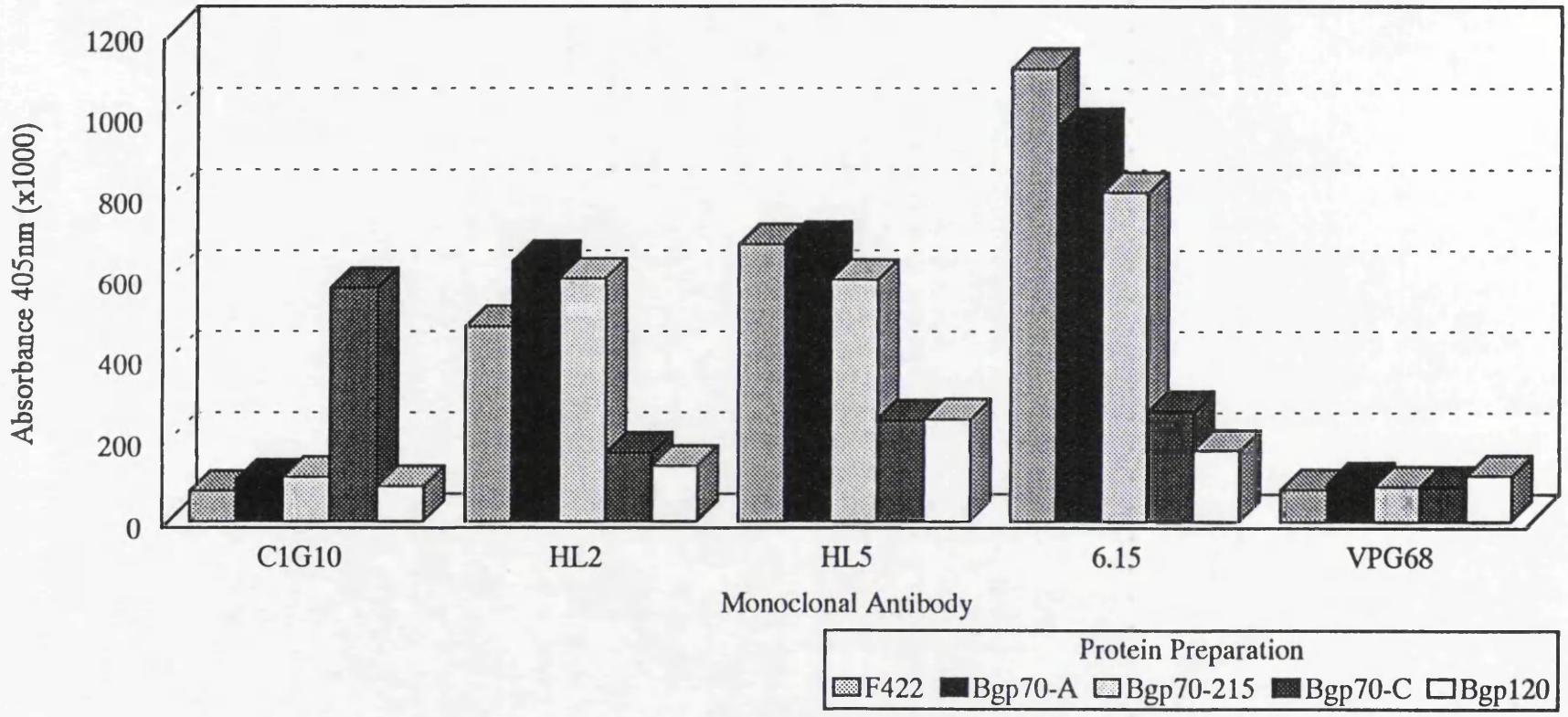
This ELISA was performed by incubating overnight several aliquots of each recombinant protein diluted with coupling buffer (100 µl per well). The quantities of the recombinant proteins used were 4 µg (Bgp100, Bgp70-A and -215) or 1.3 µg (Bgp70-C). The next day the plate was blocked with 2% Marvel and 10% goat serum in TBS (200 µl per well). Those antibodies that were only available in ascites form (6-15, HL2 and HL5) were diluted 1 in 200 with 2% Marvel and 10% goat serum in TBS. The purified antibodies (VPG 68 and C1G10) were diluted 1 in 100 from 1 mg/ml stocks. The diluted monoclonal antibodies were then added to the wells in a chequerboard fashion and the plate was incubated for 2 hours. The plate was then incubated with a 1 in 1,000 dilution (in 2% Marvel and 10% goat serum in TBS) of a goat anti-mouse IgG - alkaline phosphate conjugate (BioRad) for 1 hour. The reaction was developed by adding 50 µl of a 1 mg/ml solution of 4-nitrophenyl phosphate in AP buffer to each well and incubating for approximately 4 hours.

The results of this experiment are presented in Figure 5.4. The monoclonal antibody C1G10 reacted well with Bgp70-C but did not react with the other Bgp70s. The monoclonal antibodies HL2 and HL5 did not react with Bgp70-C but reacted well with the other Bgp70s. The monoclonal antibody 6-15 reacted with all three Bgp70s, but the reaction with Bgp70-C was weak.

5.2.3 Neutralisation Inhibition Assays

To investigate the ability of neutralising antibodies to bind to the recombinant proteins a series of experiments was performed which were referred to as neutralisation inhibition assays (NIAs). These assays measured the ability of the recombinant proteins to increase the apparent titre of virus in the presence of virus

Figure 5.4 Reaction of recombinant proteins with subgroup-specific monoclonal antibodies



To investigate the ability of the subgroup-specific monoclonal antibodies, C1G10, HL2 and HL5, to bind to the recombinant proteins aliquots of the proteins, diluted with coupling buffer, were incubated overnight in an ELISA microtitre plate. The next day the plate was blocked and then was probed with aliquots of the monoclonals, or aliquots of the control monoclonal antibodies, 6-15 and VPG 68. After 2 hours, the plate was incubated with a goat anti-mouse IgG - alkaline phosphate conjugate (BioRad) for 1 hour. The assay was developed with 4-nitrophenylphosphate.

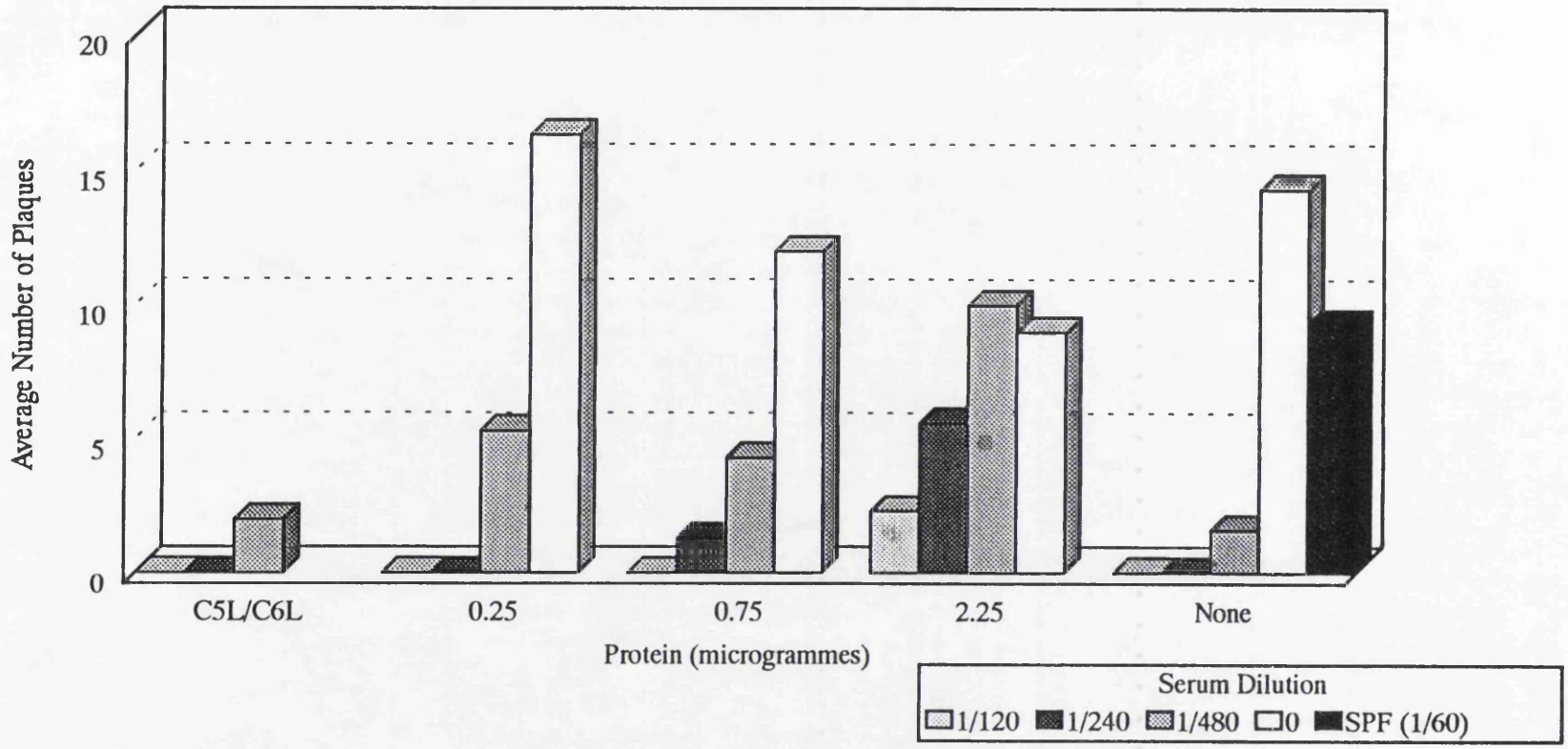
neutralising antibodies. The ability of a recombinant protein to block the action of virus neutralising antibodies was taken to indicate that the neutralising antibody was binding to the protein. As these assays were potentially susceptible to a wide range of non-specific or spurious reactions, a number of controls were included. The assays were performed using QN10S cells seeded the previous day in 12-well tissue culture trays at a density of 4 x 10^4 cells per well in 1 ml of medium containing 4 μg/ml of polybrene. Each assay was performed at least twice on separate days to reduce the likelihood of spurious reactions.

Initially, the amount of Bgp70-A required to block the action of neutralising antibodies was titrated. Aliquots of 50 μl of dilutions of Bgp70-A and P11 serum were mixed in a chequerboard fashion in a sterile, round bottomed 96-well microtitre tray. An aliquot of FeLV-A/Glasgow-1 was diluted with medium until a titre of 2.4 x 10^3 pfu/ml was obtained. From this diluted stock further aliquots of 50 μl containing 120 pfu were added to the reactions. The reactions were then incubated for 2 hours at 37°C in a humidified incubator. Three aliquots of 25 μl were taken from each reaction and were used to infect 3 wells of QN10S cells. The reaction inocula were incubated with the cells for a further 90 minutes. After this interval, the overlying medium was replaced with fresh medium and the cells were incubated for a further 6 days before the number of plaques were counted microscopically.

To control for direct interaction of the virus and the antigen, some reactions were performed without serum. To demonstrate that the P11 cat serum contained virus neutralising antibodies, other reactions were performed without recombinant protein. To establish that the recombinant proteins were not acting with non-specific components of the cat P11 serum to enhance viral entry, some reactions were performed with SPF cat serum. To control for non-specific cytotoxicity of the antigen, some wells had only the proteins added. Non-specific inhibition of neutralising activity by baculoviral or insect cell proteins was controlled by inclusion of reactions with a mixture of the two negative controls (C5L and C6L) in place of the recombinant protein. When one component of the reaction was omitted fresh medium was used in its place.

The results of this experiment are presented in Figure 5.5. A significant, specific inhibition of neutralising activity was demonstrated. The degree of variability in the average number of plaques in the absence of serum and protein (+/- 20%) was a common, though not universal, finding in these experiments. No reason was determined for this variability. The decrease in the average number of plaques observed with the SPF serum was taken as the lower limit of this variability. The negative protein control (C5L/C6L) did not block the action of neutralising

Figure 5.5 Neutralisation inhibition assay 1: titration of recombinant protein Bgp70-A



To investigate the ability of the recombinant Bgp70-A protein to specifically block the action of neutralising serum, dilutions of the P11 immune cat serum were incubated with dilutions of the protein and a fixed dose of virus for 2 hours. Aliquots from this reaction were then used to infect QN10S cells. The number of plaques produced were counted 7 days later. Controls included; no protein ('None' above), a mixture of wild type and uninfected supernatants prepared in a similar fashion to Bgp70-A (C5L/C6L above), and an SPF serum ("Wendy").
The greater the number of plaques, the greater the inhibition of the neutralising antibodies.

antibodies. Neutralising activity was not observed in the SPF serum (even at dilutions as low as 1 in 4 - data not shown). The cells did not undergo any changes in morphology in the presence of the protein alone.

In the absence of serum, increasing quantities of recombinant protein appeared to cause a decrease in the number of plaques. This suggested that, to a slight extent, the protein might have directly inhibited the replication of the virus. However, this particular result was not obtained in subsequent experiments. Moreover the number of plaques obtained with the SPF serum was always lower, indicating that normal experimental error was probably responsible for this apparent decrease.

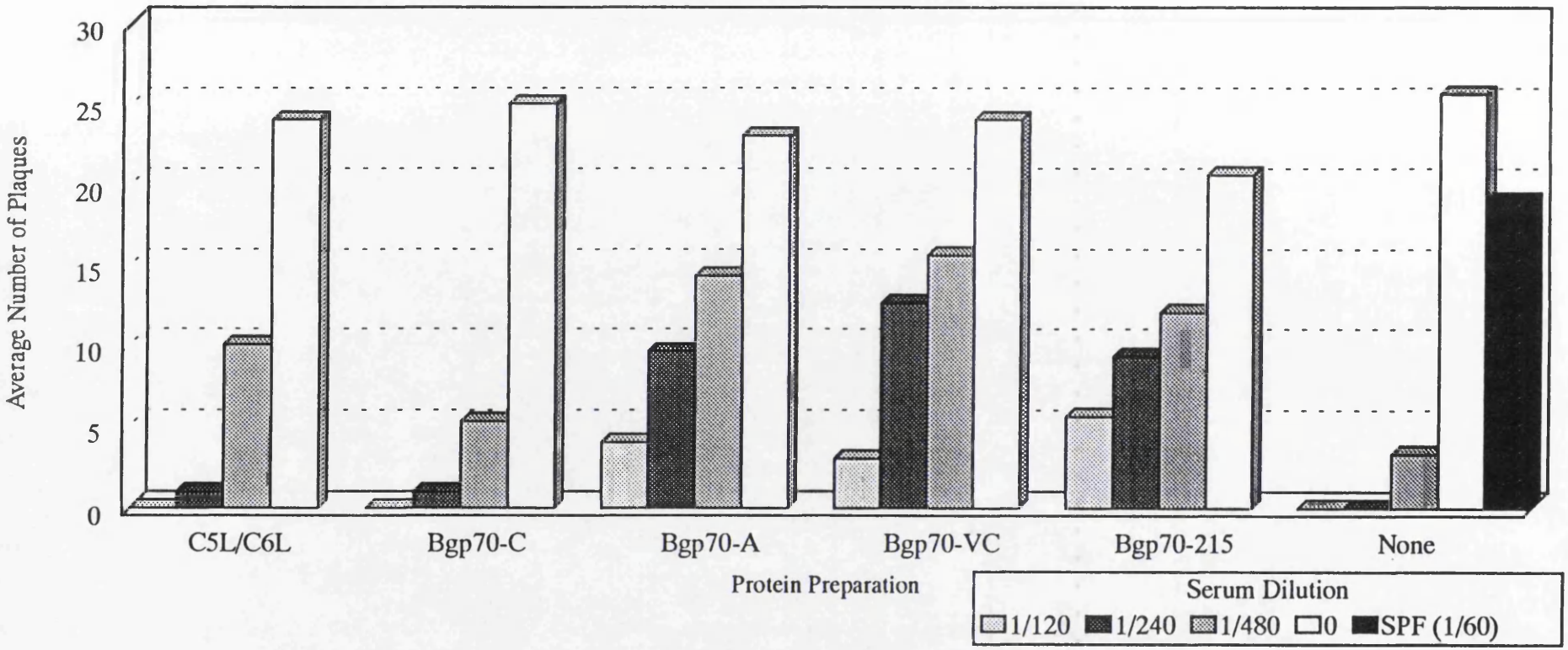
The second neutralisation inhibition assay investigated the ability of the other Bgp70s to block the action of neutralising antibodies. The assay was performed in the same manner as that described above with the sole exception of the inclusion of different recombinant protein preparations in the place of the dilutions of Bgp70-A. The results are presented in Figure 5.6 (A). Inhibition of neutralising activity was observed with Bgp70-A, Bgp70-VC and Bgp70-215. No inhibition was observed with Bgp70-C, the negative controls or the controls without protein. This result suggested that most of the neutralising activity of P11 serum was directed at regions other than that which determined subgroup phenotype. This assay was repeated with the P12 serum that is known to be specific for FeLV-A. The result was similar (see Figure 5.6 (B)). This result implied that the P12 serum was not unusual in its FeLV-A specificity.

5.2.4 Deglycosylation of Bgp70-A

Insect cell glycosylation differs both qualitatively and quantitatively from that of its mammalian counterparts (Luckow and Summers, 1988). To investigate the nature of the recombinant proteins' glycosylation, an endoglycosidase was used to remove all the N-linked carbohydrate side chains which were attached to the specific recognition sequence Asn-X-Ser/Thr. The recombinant protein was denatured before incubation with the enzyme as this raises the susceptibility of the protein to deglycosylation.

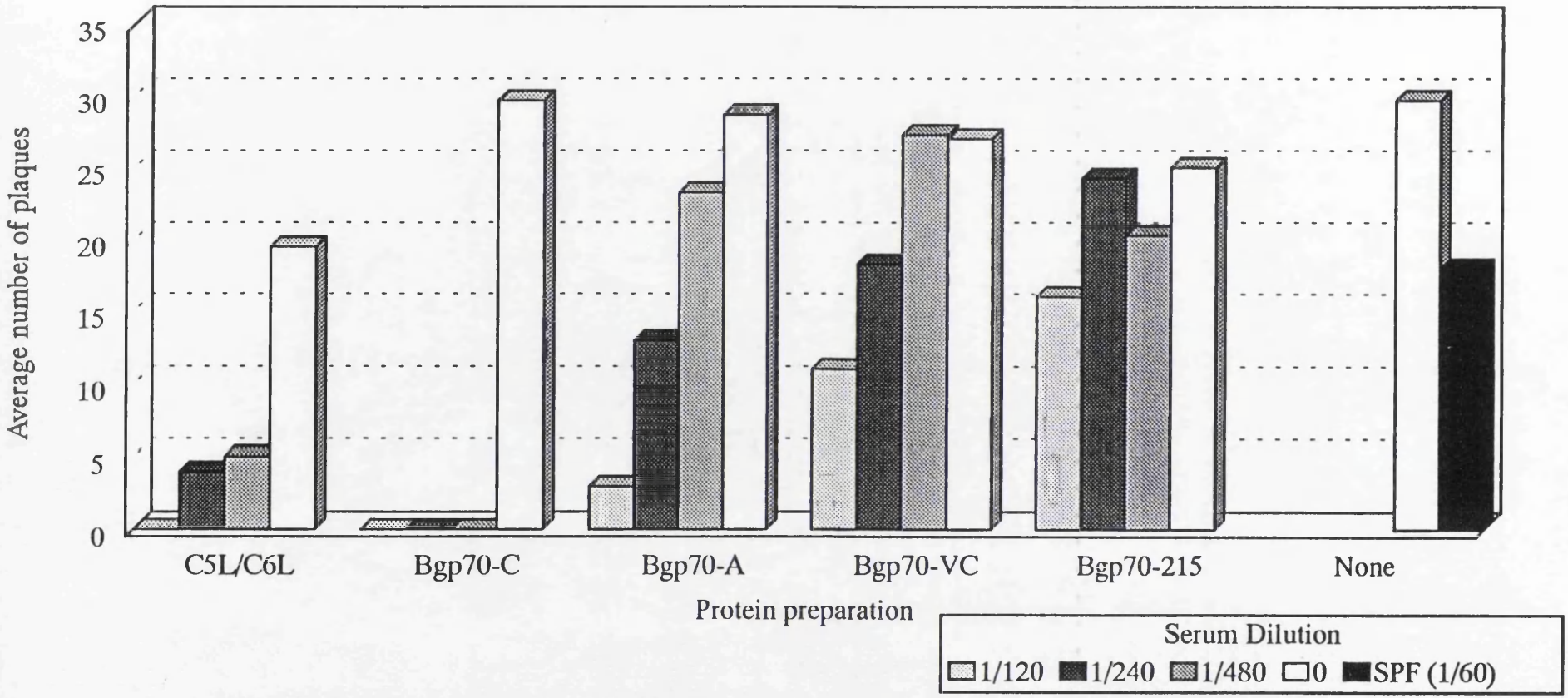
An aliquot of 160 µl of Bgp70-A containing 16 µg total protein was added to 12.5 µl β mercaptoethanol, 50 µl E2 buffer (Oxford Glycosystems) and 12.5 µl 10% SDS. The solution was boiled for 2 minutes, then centrifuged at 6,000 rpm for 30 seconds in a benchtop centrifuge. An aliquot of 40 µl of 20% Triton X100 was added and the solution was incubated at room temperature for 5 minutes. A 30 µl sample was taken and frozen with 15 µl of SDS gel loading buffer. Then 2 units (10 µl) of the enzyme peptide-N-glycosidase-F (PNGaseF) (Oxford Glycosystems) was added to the solution together with 10 µl of E2 buffer and 0.1 µg of pepstatin. The

Figure 5.6 (A) Neutralisation inhibition assay 2: reaction of four recombinant proteins with P12 serum



To investigate the ability of the recombinant Bgp70 proteins to specifically block the action of an FeLV/A specific neutralising serum, dilutions of the P12 immune cat serum were incubated with 2.25μg of the proteins and a fixed dose of virus for 2 hours. Aliquots from these reactions were then used to infect QN10S cells. The number of plaques produced were counted 7 days later. Controls included; no protein ('None' above), a mixture of wild type and uninfected supernatants prepared in a similar fashion to Bgp70-A (C5L/C6L above), and an SPF serum, Q25.
The greater the number of plaques, the greater the inhibition of the neutralising antibodies.

Figure 5.6 (B) Neutralisation inhibiton assay 3: reaction of four recombinant proteins with P11 serum



To investigate the ability of the recombinant Bgp70 proteins to specifically block the action of a neutralising serum, dilutions of the P11 immune cat serum were incubated with 2.25µg of the proteins and a fixed dose of virus for 2 hours. Aliquots from these reactions were then used to infect QN10S cells. The number of plaques produced were counted 7 days later. Controls included; no protein ('None' above), a mixture of wild type and uninfected supernatants prepared in a similar fashion to Bgp70-A (C5L/C6L above), and an SPF serum, Q25.
The greater the number of plaques, the greater the inhibition of the neutralising antibodies.

reaction was incubated for 24 hours at 25°C. Aliquots of 30 µl were taken 10, 20, 40, 70, 140, 240 minutes and 24 hours later. Each aliquot was immediately placed in 15 µl of SDS gel loading buffer and then frozen.

A similar procedure was performed with concentrated, purified FeLV from F422 supernatant. All the samples were boiled before loading on a 7.5% SDS-polyacrylamide gel. Electrophoresis was continued for 4 hours at 200 V. The proteins were then electroblotted onto a nylon membrane and probed with a 1 in 600 dilution of 3-17AP. The western blot was then developed with NBT/BCIP in AP buffer.

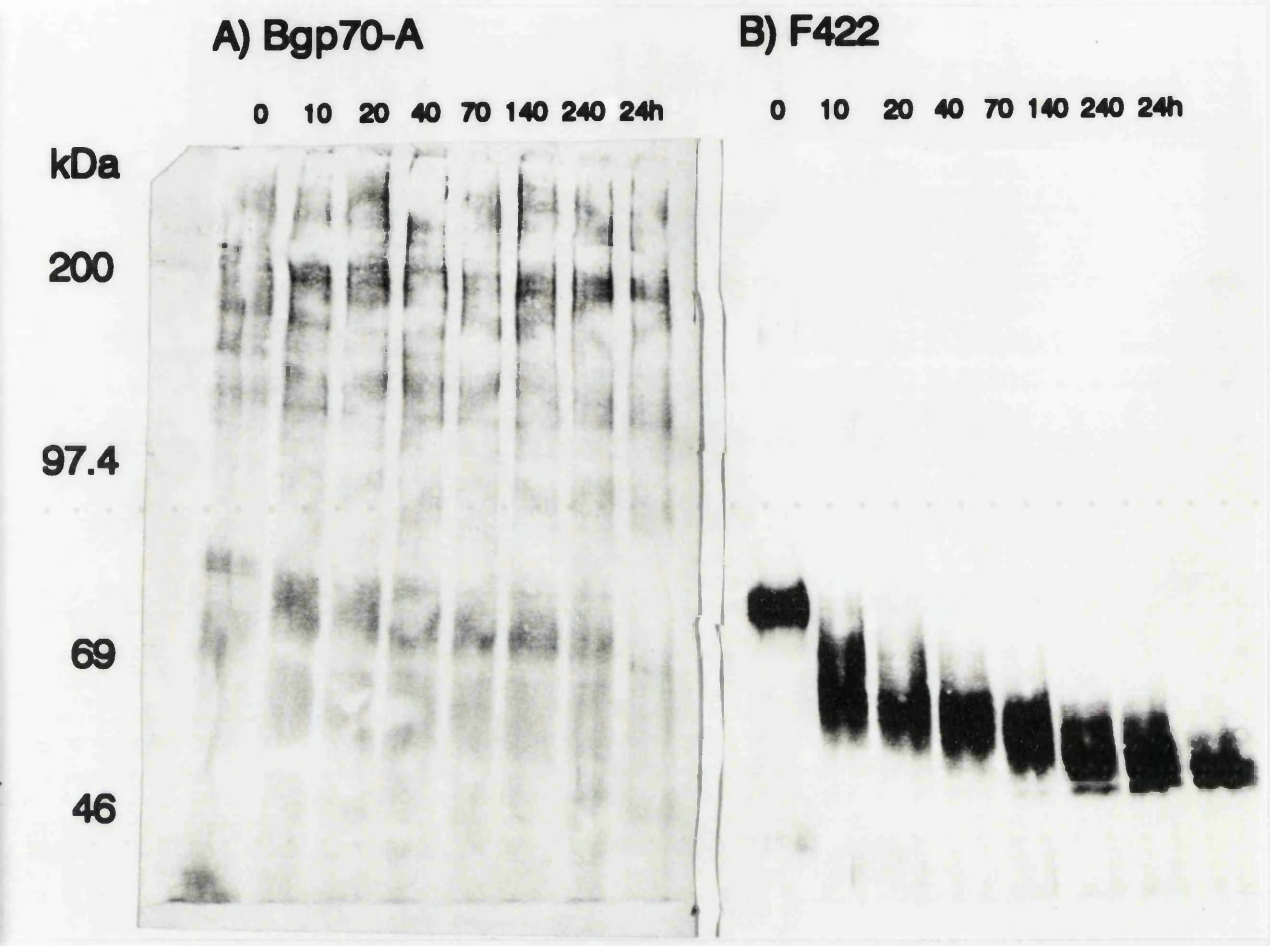
The results of this experiment are presented in Figure 5.7. It could be seen that the F422 derived gp70 was effectively deglycosylated with several intermediates. The final product was approximately 50 kDa in size, which is close to the predicted non-glycosylated size of 45 kDa. The difference was probably due to the small quantity of carbohydrate attached to serine or threonine residues (O-linked) which are not cleaved by the enzyme. Such O-linked carbohydrate side chains comprise approximately 10% of the total carbohydrate content of native FeLV SU (Pinter and Honnen, 1988). In contrast the final product of the deglycosylation of Bgp70-A was approximately 65 kDa in size, which again is slightly more than the predicted size of 60 kDa. It is known that insect cells can perform O-linked glycosylation (King and Possee, 1992). Unfortunately, the intermediates in the deglycosylation of Bgp70-A were not readily distinguishable. Therefore it was not possible to confirm that all the glycosylation sites used in native FeLV SU were also used in Bgp70-A. The high molecular mass forms of Bgp70-A were not affected by deglycosylation, suggesting that their presence was not due to aberrant glycosylation.

5.3 Discussion

The experiments in this chapter examined the similarities and differences exhibited by the four recombinant FeLV SU proteins, Bgp70s. It was found that the baculovirus expression system had produced Bgp70s that were immunologically similar to the native proteins. Furthermore, using subgroup-specific antibodies, one of the recombinant proteins could be distinguished. The two chimeric proteins were, however, indistinguishable from Bgp70-A.

The results obtained from the western blots demonstrated that the monoclonal antibody 3-17AP bound to all four recombinant proteins as did the rabbit anti-gp70 serum, #709. The P11 cat serum also bound to all four recombinant proteins, indicating that it possessed antibodies that recognised linear epitopes on all the Bgp70s.

Figure 5.7. Deglycosylation of Bgp70-A and native gp70



Aliquots of Bgp70-A and F422 virus were boiled with 0.1% b mercaptoethanol and 1% SDS for 2 minutes. Triton X 100 was added to the denatured proteins which were then incubated for an extended period with PNGase F, the appropriate buffer and the protease inhibitor, pepstatin. Aliquots were taken at various time points (0, 10, 20, 40, 70, 140, 240 minutes and 24 hours) and immediately frozen. The samples were separated by SDS-PAGE and then were transferred onto a nylon membrane. The blot was probed with 3.17AP and each side was developed separately with NBT and BCIP.

The relatively poor ability of the #709 / 3-17AP sandwich ELISA to react with native gp70 was not investigated. The ability of the recombinant Bgp70s to react well in the same assay was the only difference that was observed in immunological reactivity between the native and recombinant proteins. Immunoreactivity has been used in the quantification of recombinant protein (Hu *et al.*, 1987). However, the difference in reactivity between the native SU and Bgp70s in this assay illustrates the care with which this technique should be used. In the second set of ELISAs the quantity of recombinant antigen required to elicit the same response as 1 μg of native gp70 was many times greater than in the #709 / 3-17AP ELISA.

The 3-17 capture / cat serum sandwich ELISA results (Figure 5.3), when examined as a group, suggested that the majority of the anti-gp70 antibodies were directed at regions other than that which determines subgroup phenotype. Unlike western blots, ELISAs preserve the structural integrity of antigens and so represent a better approximation to the 'natural' interaction. The ability of the cat 31 serum to distinguish Bgp70-C from Bgp70-A suggested that the predominant epitope it recognised may be subgroup specific. However, the A/C-Sarma chimera Bgp70-VC was not recognised by the cat 31 serum. As this is derived from a chimeric virus which in superinfection interference assays is classified as belonging to subgroup C (Rigby, 1989) it therefore follows that the cat 31 serum recognises a component of FeLV-C/Sarma SU which is unique to FeLV-C/Sarma, but is not the subgroup-determining VR1 region. The reciprocal experiment with the FeLV-A specific P12 cat serum demonstrated that cats which recover naturally from FeLV infection do not generate antibodies that recognise the subgroup-determining VR1 region. This result was repeated with P11 cat serum to demonstrate that P12 serum was not an unusual variant.

The neutralisation inhibition assay results extended these observations by demonstrating that the majority of the neutralising antibodies were directed at regions other than those which determined subgroup phenotype. It was not possible to perform a NIA with cat 31 serum as only very limited supplies were available. Using Bgp70-C it might be possible to generate a serum similar to that of cat 31. The differences in reactivity of cat 31 serum and P12 serum are of importance when attempting to identify neutralising epitopes on FeLV SU.

These results are all in accordance with previously published work on the specificity of neutralising antibodies in relation to subgroup phenotype (Russell and Jarrett, 1978a; Russell and Jarrett, 1978b). The present work, in utilising subgroup chimeras and isolated recombinant SUs, has confirmed that the regions of SU that are responsible for subgroup specification are distinct from the regions that are recognised by neutralising antibodies. It has also confirmed that the SU proteins

can be separately classified according to their ability to bind different neutralising sera. There is a lack of correlation between subgroup phenotype based on superinfection interference and neutralisation assays (Russell and Jarrett, 1978b). The ability to produce recombinant FeLV SU which will block the neutralising activity of serum from cats that have recovered from a natural challenge represents a useful tool for further studies directed at identifying those epitopes that are recognised by such virus neutralising antibodies. As the majority of neutralising antibodies are specific to a neutralisation subgroup the epitope recognised by 3-17 is unlikely to be one of the epitopes that the cat mounts a neutralising response against.

Glycosylation is important for protein solubility and, sometimes, antigenicity. The most significant disadvantage of the baculovirus expression system is that insect cell protein glycosylation differs, both quantitatively and qualitatively, from that of mammalian counterparts. The nature of glycosylation in insect cells has only been examined indirectly; by the incorporation of radiolabelled sugars, by the examination of the effect of endoglycosidases and by the addition of glycosylation inhibitors such as tunicamycin. The glycosylation of recombinant proteins manufactured in the baculovirus expression system has been best studied in the production of fowl plague haemagglutinin (Kuroda *et al.,* 1990). These studies involved cleavage of radiolabelled carbohydrate side chains with specific endoglycosidases. In mammalian cells, carbohydrates are attached to proteins by means of a precursor consisting of glucose, mannose and n-acetyl glucosamine residues on a dolichol pyrophosphate carrier. This is then trimmed and elongated in a series of species and site-specific steps. Insect cells are believed to synthesize similar precursors and trim them back to an oligomannosidic core but appear not to elongate the trimmed precursor (Kuroda *et al.,* 1990). However, they are able to introduce fucose residues onto some of their truncated carbohydrate side chains (Kuroda *et al.,* 1990).

There have been few studies on the glycosylation patterns of recombinant retroviral proteins produced in the baculovirus expression system. Recombinant ALV gp85, in contrast to wild type ALV gp85, is digestible with endoglycosidase H (Noteborn *et al.,* 1990). This result indicates that full maturation to the complex oligosaccharide structure of wild type ALV gp85 is not achieved. This may have had functional implications as inoculation of chickens with crude lysates of the gp85-expressing Sf9 cells failed to elicited neutralising antibodies although specific, non-neutralising, antibodies were produced. In studies of the glycosylation of recombinant HTLV-1 *env* it was demonstrated that only some of the product was glycosylated as treatment with tunicamycin did not affect the size of the major

product (Arp *et al.,* 1993).

Incomplete glycosylation does not necessarily produce a reduction in the ability to elicit neutralising antibodies. The F glycoprotein of human parainfluenza virus type 3, when produced as a recombinant protein in the baculovirus expression system, is incompletely glycosylated, yet still elicits a neutralising antibody response in cotton rats (Hall *et al.,* 1991). However in other studies carbohydrate side chains have been shown to shield antigenic determinants and prevent receptor binding (Deom *et al.,* 1986; Robertson *et al.,* 1985).

The high molecular mass species in Bgp70 preparations, seen particularly after denaturation prior to deglycosylation, may represent insoluble fractions. This could be confirmed by preparing gels containing 6 M urea which increases protein solubility. The requirement for urea to solubilise recombinant retroviral proteins for SDS-PAGE analysis has already been noted (Arp *et al.,* 1993). Further deglycosylation experiments, using more specific endoglycosidases, should be performed to assess the specific differences between native and recombinant retroviral proteins. The role of the carbohydrate side chains of FeLV SU in shielding important epitopes and in cell surface receptor binding could be investigated using the products of such experiments. In particular, PNGaseF treated Bgp70-A could be used in neutralisation inhibition assays. Other forms of denaturation, such as heat treatment, could also be used to assess the contribution of higher orders of structure on the binding of neutralisation antibodies to the recombinant SUs.

In conclusion, the recombinant surface glycoproteins of FeLV were found to be immunologically similar to their native counterparts. They inhibited the action of neutralising antibodies and did so in a subgroup specific fashion. These data suggested that the proteins may be able to act as ligands for FeLV cell surface receptors. They also suggested that the Bgp70-A protein could serve as an FeLV vaccine. These possibilities were investigated in experiments detailed in the next two chapters.

Chapter 6. Receptor Binding Studies

6.1 Introduction

This chapter describes the use of recombinant FeLV surface glycoproteins as ligands in binding studies aimed at identifying FeLV cellular receptors. The identification of the FeLV cellular receptors may help to unravel some of the mechanisms underlying the pathogenesis of retrovirus-induced disease. In particular, the unique disease specificity of FeLV-C may be due to a specific interaction of FeLV-C surface glycoprotein with early erythropoietic progenitor cells. An analogous situation has been described in the pathogenesis of murine erythroleukaemia induced by Friend spleen focus forming virus (Li *et al.,* 1990).

Recently a protein of approximately 70 kDa has been suggested as a putative receptor for FeLV-A (Ghosh *et al.,* 1992). These workers used native FeLV SU in several assays in an attempt to define the receptor. The protein was prepared by immunoaffinity chromatography of large quantities of detergent disrupted virions derived from the concentrated culture fluid of AH927 cells (Rasheed and Gardner, 1980) infected with FeLV-A. Initially the ability of the protein to bind to the feline T cell line, 3201B, was demonstrated. Binding studies were also performed with the human T cell line, HuT 78 (Gazdar *et al.,* 1980). The values obtained with this cell line were taken as the non-specific background level. No attempt was made to block virus infection directly with this preparation of FeLV SU. Subsequently, the radiolabelled FeLV gp70 was used to co-immunoprecipitate a cell surface protein with an apparent molecular mass of 70 kDa. The converse of this experiment was also performed; using ^{125}I surface labelled 3201B cells or cell membranes, a protein of similar size was co-immunoprecipitated. Control experiments were also performed with HuT 78 cells. No protein was co-immunoprecipitated in these cases.

In this chapter, an infection interference assay is described. This assay was performed to investigate the ability of one of the proteins, Bgp70-A, to block directly the infection by FeLV of a susceptible cell line. The assay was then repeated with a wider range of the recombinant proteins to analyse the specificity of the reactions.

On the basis of the results obtained from the infection interference assays, cell binding experiments were performed with detergent lysates made from ^{125}I surface labelled cells. The complexes which formed between the labelled and recombinant proteins were cross-linked and then co-immunoprecipitated with an anti-gp70 monoclonal antibody immobilised on protein G beads. As the recombinant protein

preparations contained large quantities of a protein, which was presumed to be baculoviral gp64, it was decided that radiolabelling of these preparations to detect binding of the recombinant surface glycoprotein to cell receptors would result in an high background of non-specific activity.

6.2 Results

6.2.1 Infection Interference Assays

Infection interference assays were performed to investigate the ability of the recombinant proteins to block FeLV infection of susceptible cells. The ability of detergent disrupted FeLV particles to interfere with infection of susceptible cells had previously been demonstrated by others (O.Jarrett, personal communication).

Several preliminary experiments were used to establish a reliable method for this assay. These experiments demonstrated that optimum interference by the recombinant FeLV-A surface glycoprotein, Bgp70-A, of FeLV-A infection was achieved when the recombinant protein was present both during and after the virus adsorption phase of infection. No detailed analysis was undertaken into effect of the length of time that the protein was incubated with the cells after virus absorption. Incubating the cells with the protein before, but not after, virus absorption was found to be a poor method of demonstrating infection interference. The lack of availability of the relatively large quantities of recombinant proteins required for these experiments precluded systematic optimisation of the assays.

Prior to performing the infection interference assays, the effect of amphotericin B (Fungizone, Gibco) on the QN10S assay was investigated. It was found that plaque formation was not impaired by the inclusion of this antifungal agent at a concentration of 2.5 μg/ml.

An infection interference assay was undertaken with Bgp70-A and isolates of all three FeLV subgroups. The recombinant FIV surface glycoprotein, Bgp100, was used as control in this experiment. To perform this experiment, QN10S cells were seeded in 12-well tissue culture plates at a density of 4 x 10^4 cells per well. The next day various aliquots of the recombinant proteins were added to 1 ml aliquots of fresh medium, which contained approximately 80 pfu of FeLV-A/Glasgow-1, FeLV-B/ST or FeLV-C/FA27C and polybrene at a concentration of 4 μg/ml. An additional control which contained no recombinant protein was included. The culture fluid was removed from the QN10S cells and replaced with 0.5 ml aliquots of the virus-protein-medium mixtures. Each assay was performed in duplicate. The cells were incubated for 2 hours, after which time the mixtures were removed and replaced with 0.5 ml aliquots of protein-medium mixtures prepared in a similar

fashion as above, but without virus. The cells were incubated for a further 2 days and a further 1 ml aliquot of fresh medium containing amphotericin B (Fungizone, Gibco) at a concentration of 2.5 μg/ml was added. The cells were maintained for a further 4 days before the number of plaques were counted. The results are shown in Table 6.1 and are compared using a graph in Figure 6.1.

A reduction in the number of plaques was observed when Bgp70-A was included in the culture fluid during and after FeLV-A infection of QN10S cells. This result suggested that Bgp70-A interfered with FeLV-A infection. The recombinant FIV SU protein, Bgp100, did not interfere with FeLV-A, FeLV-B or FeLV-C infections. Furthermore, Bgp70-A did not interfere with FeLV-B or FeLV-C infections. This result suggested that the interference observed between Bgp70-A and FeLV-A was subgroup specific. The failure to completely inhibit the infection of the cells was believed to be a dose-dependant phenomenon, though an absolute inability of the protein to completely inhibit the infection could not be discounted.

In the control reactions a slight rise in the number of plaques was observed with increasing volume of recombinant protein added. The rise was constant between the various controls and was not dependent on the FeLV subgroup used. A non-specific interaction between FeLV and baculoviral proteins may account for this observation. Alternatively, the slight decrease in serum protein concentration, caused by adding the recombinant protein solution to a maximum volume of 50 μl to the original 0.5 ml aliquot of viral inoculum, might be responsible.

To extend the above observations, another infection interference assay was performed with a larger range of proteins. The assay was similar to that described above. The culture fluid of QN10S cells was replaced with 0.5 ml aliquots of FeLV-A inocula to which had been added polybrene and various concentrations of one of the recombinant proteins, Bgp70-A, Bgp70-C, Bgp70-215 or Bgp100. A control mixture without added protein was included. The control protein preparation C5L, which was prepared from the culture fluid of uninfected Sf9 cells (see section 4.2.3), was also incorporated into this assay. The cells were incubated with the virus-protein-medium mixtures for 2 hours and then the mixtures were removed and replaced with 0.5 ml aliquots of medium containing the recombinant proteins, but without virus. The cells were incubated for a further 2 days and a further 1 ml aliquot of fresh medium containing amphotericin B was added. The cells were maintained for a further 4 days before the number of plaques were counted. The results are shown in Table 6.2 and are compared using a graph in Figure 6.2 (A).

The specific interference of FeLV-A infection by Bgp70-A, previously observed in

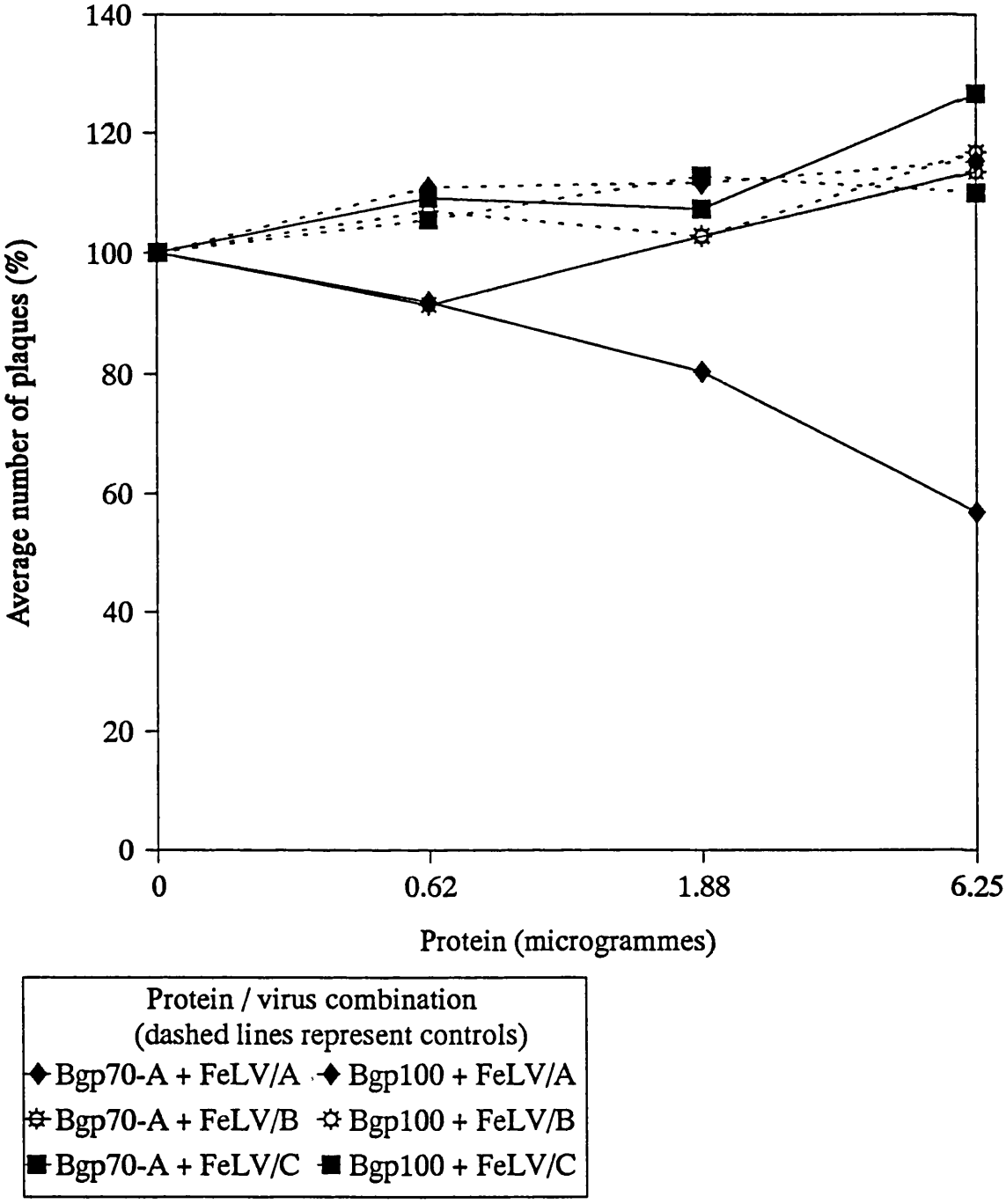
Table 6.1 Infection interference assay 1: titration of protein

1. Bgp70-A	Virus		
Amount of protein (μg)	FeLV-A/Glasgow-1	FeLV-B/ST	FeLV-C/FA27C
None	71.25	61.25	54.5
0.62	65.5	56	59.5
1.88	57.5	63	58.5
6.25	43	69.5	69

2. Bgp100	Virus		
Amount of protein (μg)	FeLV-A/Glasgow-1	FeLV-B/ST	FeLV-C/FA27C
None	71.25	61.25	54.5
0.62	79	63.5	57.5
1.88	79.5	63	61.5
6.25	81.5	71.5	60.5

The above tables show the results obtained from an infection interference assay, which was performed by incubating QN10S cells with a 0.5 ml aliquot of either diluted virus alone, or virus with recombinant protein added. After 2 hours, the inocula were removed and replaced with either medium alone or medium with recombinant protein added. Two days later, a further 1 ml volume of medium was added. Each assay was performed in duplicate. The average number of plaques from the 2 wells is shown (4 wells were used for the controls without added recombinant protein). The values produced were further expressed as a fraction of the values obtained from the controls without protein, the results are presented in this manner in Figure 6.1.

Figure 6.1 Infection interference assay 1: titration of protein



The following experiment was performed to determine whether Bgp70-A could interfere with viral infection of susceptible cells and if such interference was subgroup-specific. QN10S cells were infected for 2 hours with a 0.5 ml aliquot either of virus alone, or of virus with recombinant protein added. The inoculum was removed and replaced with a 0.5 ml aliquot either of medium alone, or of medium with recombinant protein added. Two days later a further 1 ml of medium was added. Each assay was performed in duplicate. The plaques were counted 6 days post-infection. The average number of plaques was expressed as a percentage of the average of number of plaques counted in controls with no protein.

Table 6.2 Infection interference assay 2: inhibition of FeLV-A

Experiment A

Quantity of protein (μg)	Protein				
	Bgp70-A	Bgp70-C	Bgp70-215	C5L	Bgp100
None	17	17	17	17	17
0.5	22	16	12.5	18	14.5
1.5	11	16.5	17	16	18.5
5	8.5	15	16.5	17	18.5
15	0	15	15.5	23	20

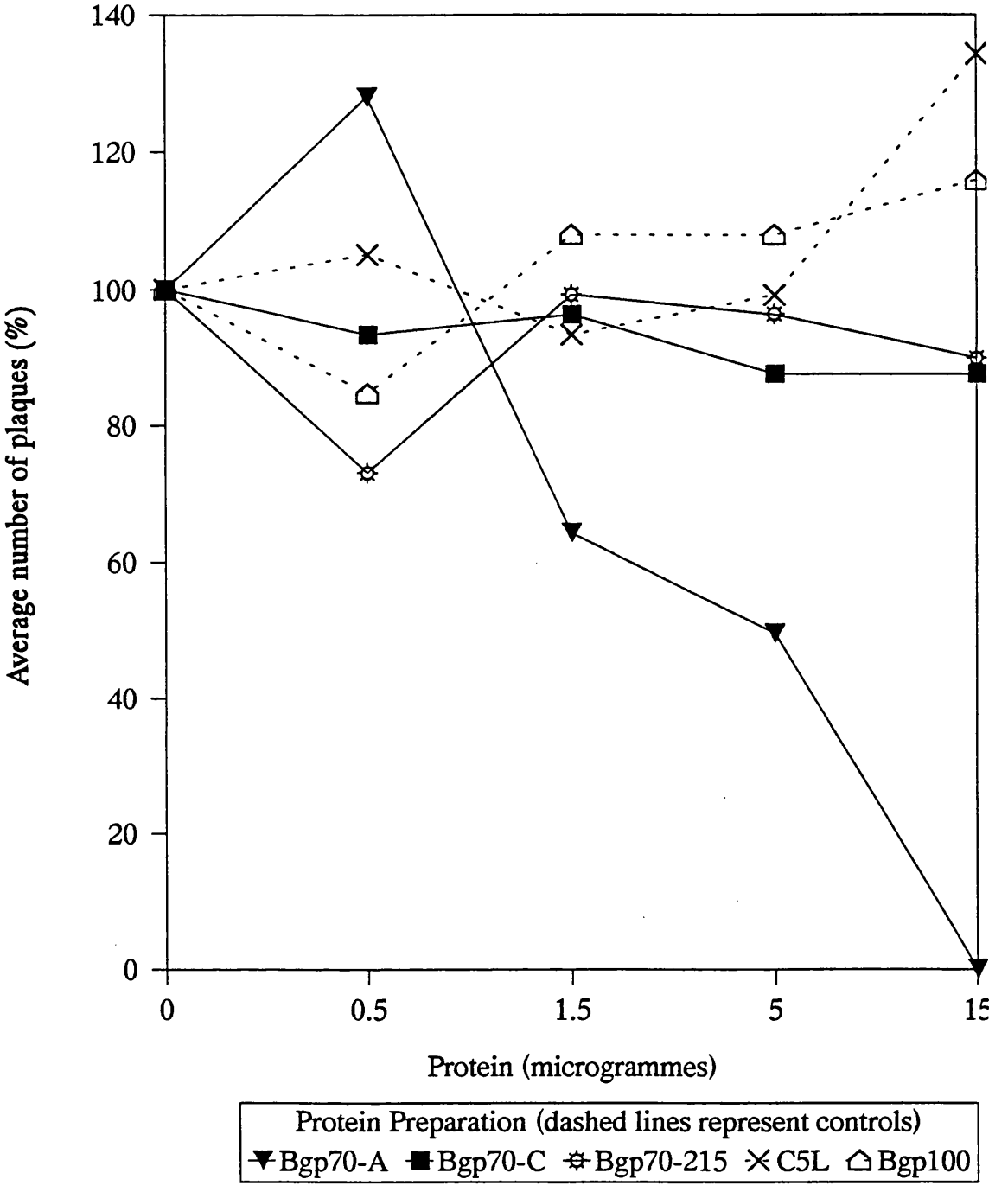
Experiment B

Quantity of protein (μg)	Protein				
	Bgp70-A	Bgp70-C	Bgp70-VC	Bgp70-215	C5L/C6L
None	46.6	46.6	46.6	46.6	46.6
0.5	NR	45.5	48	51	53.5
1.5	50.5	47	56.5	64	53.5
5	20.5	61.5	66	62	42
15	0	64	64.5	71.5	60

These tables show the results obtained from infection interference assays performed with a range of the recombinant proteins and FeLV-A. These assays were performed by incubating QN10S cells with a 0.5 ml aliquot of either the diluted virus alone, or virus with recombinant protein added. After 2 hours, the inocula were removed and replaced with either medium alone or medium with recombinant protein added. Two days later, a further 1 ml volume of medium was added. Each assay was performed in duplicate. The average number of plaques from the 2 wells is shown (8 wells were used for the controls without added recombinant protein). The values produced were further expressed as a fraction of the values obtained from the controls without protein, the results are presented in this manner in Figure 6.2 (A) and (B).

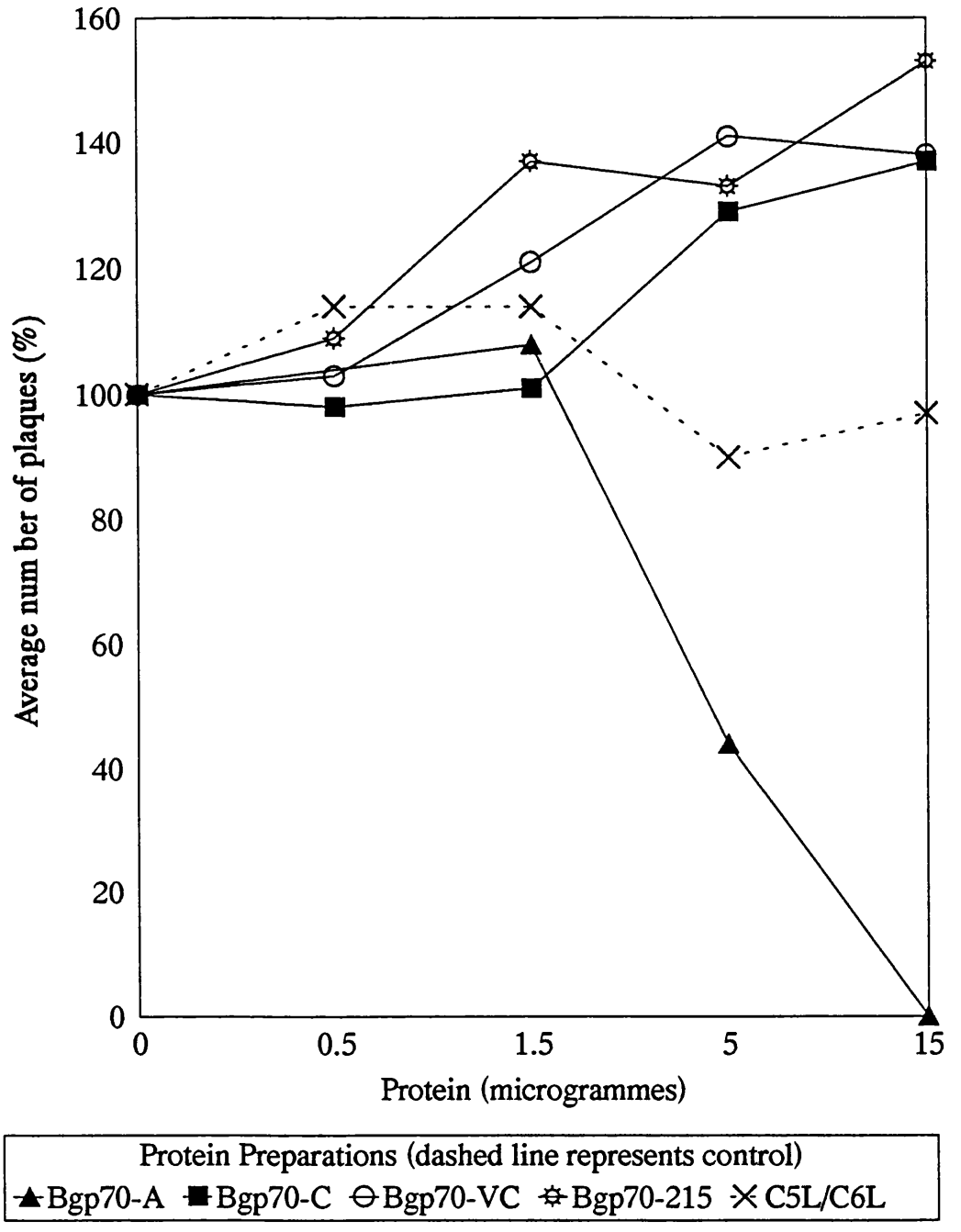
NR = No result (fungal contamination)

Figure 6.2 (A) Infection interference assay 2: inhibition of FeLV-A



The following experiment was performed to investigate the ability of the recombinant proteins to interfere with the infection of susceptible cells by FeLV-A/Glasgow-1. QN10S cells, seeded in a 12-well tray the previous day, were incubated with a 0.5 ml viral inoculum containing a diluted recombinant protein for 2 hours. After this period the inoculum was removed and replaced with 0.5 ml of fresh medium containing only the protein. After 2 days a further 1 ml of fresh medium was added and the cells were incubated for a further 5 days. Each assay was performed in duplicate. The plaques were counted 7 days post-infection. The average number of plaques was expressed as a percentage of the average of number of plaques counted in controls with no protein.

Figure 6.2 (B) Infection interference assay 2: inhibition of FeLV-A



The following experiment was performed to investigate the ability of the recombinant proteins to interfere with the infection of susceptible cells by FeLV-A/Glasgow-1. QN10S cells, seeded in a 12-well tray the previous day, were incubated with a 0.5 ml viral inoculum containing a diluted recombinant protein for 2 hours. After this period the inoculum was removed and replaced with 0.5 ml of fresh medium containing only the protein. After 2 days a further 1 ml of fresh medium was added and the cells were incubated for a further 5 days. Each assay was performed in duplicate. The plaques were counted 7 days post-infection. The average number of plaques was expressed as a percentage of the average of number of plaques counted in controls with no protein.

Figure 6.1, is again demonstrated in Figure 6.2. Neither Bgp100 nor C5L interfered with the infection of susceptible cells. Significantly, Bgp70-C and Bgp70-215 failed to inhibit the infection of the cells by FeLV-A. This result confirmed the subgroup-specific nature of the infection interference caused by the recombinant proteins.

The infection of the cells was completely inhibited at the highest dose of Bgp70-A. The wide range in the number of plaques counted in some of the wells that contained control protein preparations was probably due to normal experimental variation. The assay was repeated with different batches of protein with similar results. See Table 6.2 and Figure 6.2 (B).

To determine if FeLV-C could be inhibited by the recombinant chimeric FeLV-A/C protein, Bgp70-215, an infection interference assay was performed with FeLV-C/FA27C and the same range of recombinant proteins used previously. The assay differed slightly from those described previously. Instead of adding different volumes of the recombinant proteins to 0.5 ml of the viral inoculum, the volume added to the FeLV-C inoculum was kept constant with the addition of sterile TBS. A control mixture without protein was included. A 50:50 mixture of C5L and C6L (see section 4.2.3) was also used in this assay. The cells were incubated with the virus-protein-medium mixtures and then the protein-medium mixtures as before. The results are presented in Table 6.3 and Figure 6.3.

Inhibition of plaque formation was observed in those wells that contained either Bgp70-C or the FeLV A/C chimeric protein, Bgp70-215. This result extended previous observations on the ability of Bgp70-A to interfere with FeLV-A infection by demonstrating that Bgp70-C interfered specifically with FeLV-C. The ability of Bgp70-215 to interfere with FeLV-C, but not FeLV-A, demonstrates the functional similarity between the A/C subgroup-determining VR1 region of the recombinant proteins and native FeLVs (Rigby, 1989).

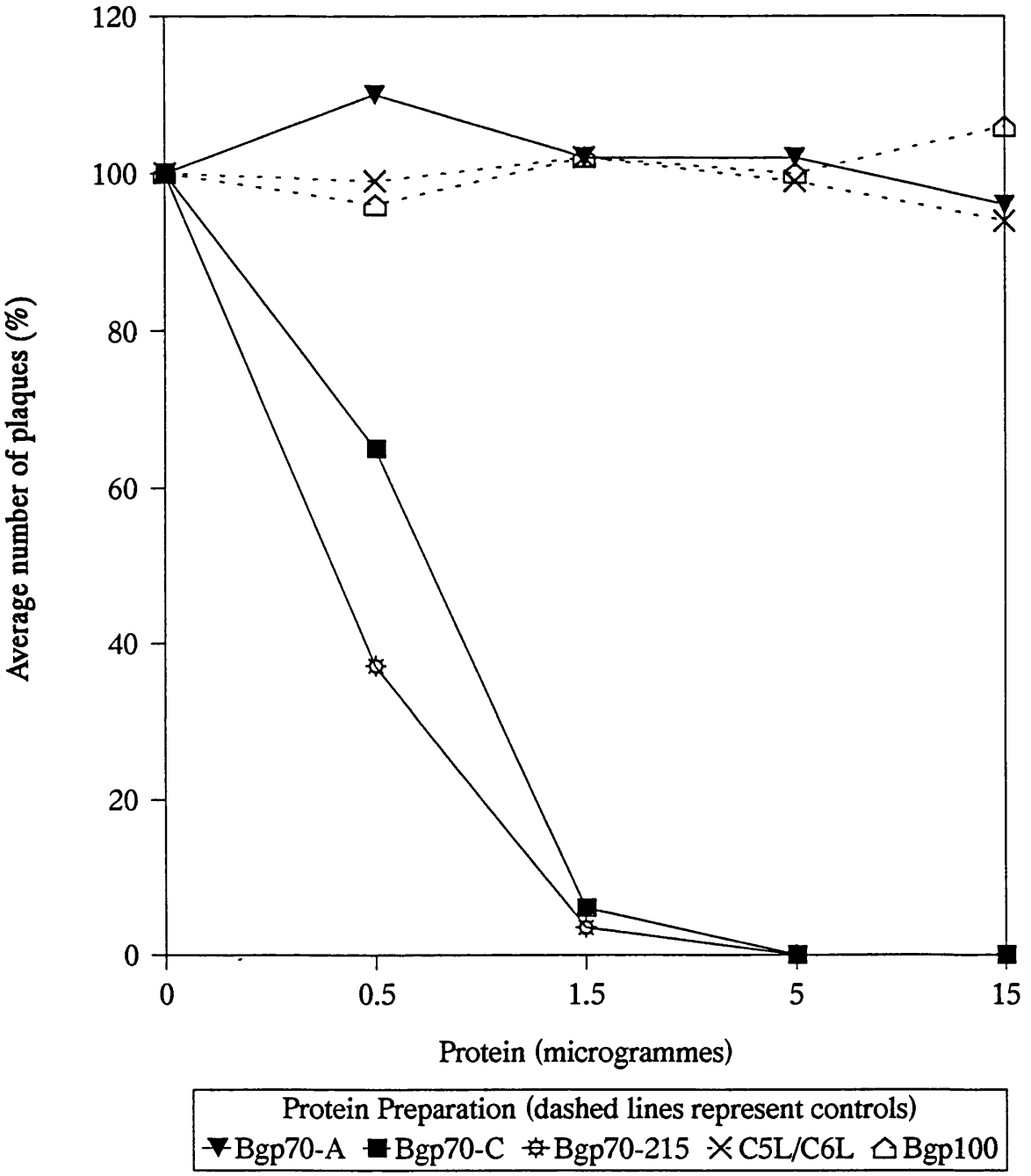
It was noted that, by keeping the volume of viral inoculum constant, the increase in plaque formation noted in Figures 6.1 and 6.2 was no longer observed. This result suggested that slight decreases in serum protein concentration slightly increase the titre of the virus. It is known that larger reductions in serum protein concentration result in decreases in viral titre in neutralisation assays (Russell, 1977). A reduction in the number of spurious variations in plaque numbers was also noted. In retrospect it would have been preferable to keep the volume of inoculum constant in these experiments. Time constraints and the lack of large quantities of recombinant proteins prevented the experiments presented in Figures 6.1 and 6.2 from being repeated with this modification.

Table 6.3 Infection interference assay 3: inhibition of FeLV-C

Quantity of protein (μg)	Protein				
	Bgp70-A	Bgp70-C	Bgp70-215	C5L/C6L	Bgp100
None	57.75	57.75	57.75	57.75	57.75
0.5	63.5	37.5	21	57	55
1.5	59	7	2	59	59
5	59	0	0	57	57.5
15	55	0	0	54	61.5

This table shows the results obtained from infection interference assays performed with a range of the recombinant proteins and FeLV-C/FA27C. The assay was performed by incubating QN10S cells with a 0.5 ml aliquot of either the diluted virus alone, or virus with recombinant protein added. After 2 hours, the inocula were removed and replaced with either medium alone or medium with recombinant protein added. Two days later, a further 1 ml volume of medium was added. Each assay was performed in duplicate. The average number of plaques from the 2 wells is shown (8 wells were used for the controls without added recombinant protein). The values produced were further expressed as a fraction of the values obtained from the controls without protein, the results are presented in this manner in Figure 6.3.

Figure 6.3 Infection interference assay 3: inhibition of FeLV-C



The following experiment was performed to investigate the ability of the recombinant proteins to interfere with the infection of susceptible cells by FeLV-C/FA27C. QN10S cells, seeded in a 12-well tray the previous day, were incubated with a 0.5 ml viral inoculum containing a diluted recombinant protein for 2 hours. After this period the inoculum was removed and replaced with 0.5 ml of fresh medium containing only the protein. After 2 days a further 1 ml of fresh medium was added and the cells were incubated for a further 5 days. Each assay was performed in duplicate. The plaques were counted 7 days post-infection. The average number of plaques was expressed as a percentage of the average of number of plaques counted in controls with no protein.

6.2.2 Co-immunoprecipitation Studies

The ability of the recombinant proteins to mimic the subgroup phenotype of the native gp70 and to block infection of susceptible cells was used in an attempt to identify the FeLV cellular receptors.

Initially the monoclonal antibody 3-17 was attached to protein G immobilised on sepharose (Sigma) and then the preparation was used to co-immunoprecipitate the putative receptor from a mixture containing the recombinant protein and an ^{125}I surface labelled cell lysate.

(1) Coupling 3-17 to Protein G

The method used to couple the monoclonal antibody 3-17 to protein G-sepharose beads was similar to a published protocol for attaching antibodies to protein A (Harlow and Lane, 1988). An aliquot of 450 µl of 3-17 ascites was mixed with 290 µl of protein G-sepharose beads and 290 µl of TBS. After 1 hour the beads were washed twice with 0.2 M sodium borate (pH 9.0) and then were resuspended in 2.9 ml 0.2 M sodium borate. Dimethyl suberimidate dihydrochloride (Aldrich) was added to a final concentration of 20 mM (=0.2 mg/ml) and the cross-linking reaction was allowed to proceed for 1 hour. The reaction was stopped by washing the beads for 2 hours in 0.2 M ethanolamine. Finally, the beads were washed twice in TBS and were stored at 4^0C.

(2) Iodination of Cells

The cell lines used in these experiments were AH927, 3201 B, MDCK and HuT 78. The materials and methods used in the culturing of these cell lines have been described previously (section 2.5). For all iodination reactions the cells were harvested at 90% confluence from 225cm^2 tissue culture flasks. The two adherent cell lines, MDCK and AH927, were harvested by incubation with a 0.025% trypsin / PBS-EDTA mixture. The cells were washed twice in PBS before being resuspended at a density of 2 x 10^7 cells/ml of PBS. Trypan blue was used to assess the viability of the cells. In all cases more than 98% cells were found to be viable.

Cell surface iodination was carried out using a commercial reagent (1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycoluril: Iodo-gen, Pierce Chemical Company) that performs a chemical oxidation reaction which labels surface tyrosine residues. Some histidine residues are also labelled using this technique (Harlow and Lane, 1988). A 100 µl aliquot of a 1 mg/ml chloroform solution of Iodo-gen was added to a 5 ml glass scintillation vial, which had been previously rinsed with chloroform. The solution was left to evaporate until a thin film of reagent could be seen coating

the glass. The vials were then stored upright in a moisture-free environment at 4^0C until required.

Immediately prior to use the vials were rinsed carefully with PBS to remove any large flakes of reagent. Aliquots of 0.5 ml of PBS and 1 mCi of $Na^{125}I$ were added, followed by a 0.5 ml aliquot of PBS containing 10^7 cells. The reaction was mixed gently before being allowed to proceed for approximately 20 minutes. The cell suspension was transferred from the vial to a 1.5 ml eppendorf tube. The cells were then precipitated by centrifugation at 6500 rpm for 1 minute in a benchtop benchtop centrifuge. The supernatant, which contained most of the unbound iodine, was carefully discarded. The cells were washed 3 times with 1 ml of PBS. Finally the cells were precipitated by centrifugation as before.

(3) Co-immunoprecipitation reactions

Experiments which investigated the interactions of Bgp70 with the cell surface proteins were performed with either cell lysates or whole cells.

(a) Cell lysates

The ^{125}I surface labelled cells were lysed by the addition of 1.2 ml of 0.5% NP40 in TBS to the precipitated cells. The lysis reaction was allowed to proceed for 5 minutes before being centrifuged at 6500 rpm in a benchtop benchtop centrifuge for 5 minutes. The protease inhibitors, leupepsin and pepstatin, were added to the supernatant to produce a final concentration of 0.5 μg/ml of each. The supernatant was frozen at -70^0C until required.

To reduce the level of non-specific binding, the lysates were pre-absorbed with a 100 μl aliquot of Sephadex G25 (Pharmacia) for 1 hour. The Sephadex G25 was removed by centrifugation at 6500 rpm for 1 minute. Aliquots of 250 μl of the lysate were incubated at room temperature for 90 minutes with 50 μl aliquots of Bgp70-A, Bgp70-C, Bgp70-215, Bgp100 which contained 25 μg of total protein. Chemical cross-linking was performed in a similar manner to that described elsewhere (Brenner *et al.,* 1985; Ghosh *et al.,* 1992). Aliquots of 3 μl from a 100 mM stock of $MgCl_2$ and 5 μl from a 10 mg/ml DMSO solution of a reducible bifunctional cross-linker, dithiobissuccinimidyl propionate (DSP), were added. The reaction was allowed to proceed for 30 minutes, then a 15 μl aliquot of 3-17-linked protein G-sepharose beads was added for 1 hour. A control reaction was performed without protein. The sepharose beads were sedimented by centrifugation at 6500 rpm for 1 minute and were then washed 3 times in PBS.

Samples were prepared for SDS-PAGE analysis by boiling the protein G-sepharose

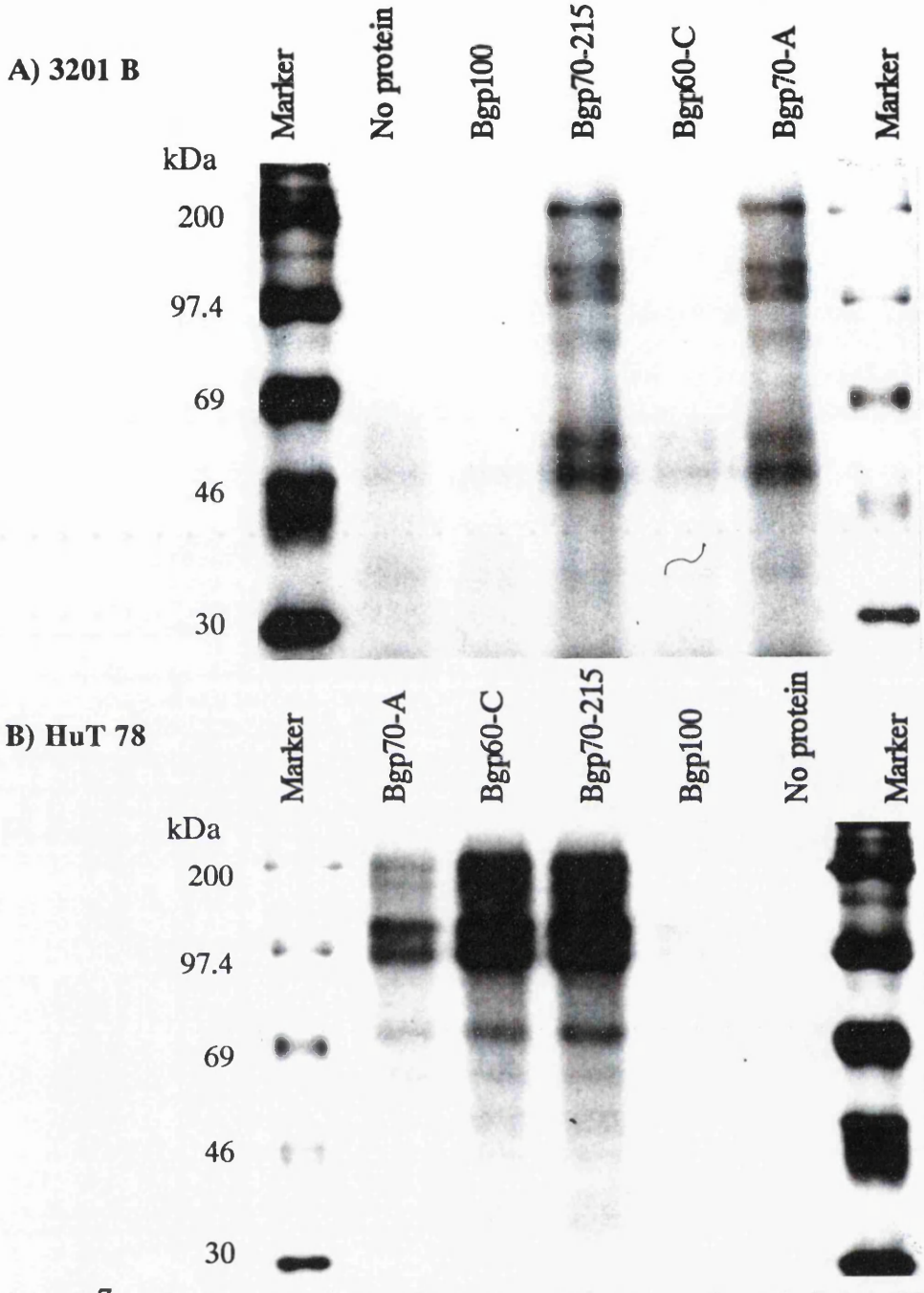
beads in 30 µl of SDS gel loading buffer and then centrifugation at 6500 rpm for 5 minutes. Aliquots of 10 µl of the resulting supernatants were loaded onto a 10% SDS-PAGE gel together with 0.5 µl ^{14}C Rainbow markers (Amersham International) + 1.5 µl Rainbow markers. The gel was run at 50 V for 2.5 hours and then fixed in de-staining solution (see section 2.3.3) for 20 minutes and enlightening solution (Du Pont) for 5 minutes. The gel was dried on Whatman 3M paper under vacuum in a heated gel dryer (Model 583, Bio-Rad) and then exposed for 7 days at -70^{0}C. Photographs of the autoradiographs are shown in Figure 6.4.

Figure 6.4 demonstrates that a number of protein species were co-immunoprecipitated from all four cell lines by this technique. No proteins were co-immunoprecipitated in the absence of recombinant protein or in the presence of a solution of Bgp100. This solution contains the same background of baculoviral proteins as those solutions containing the recombinant FeLV glycoproteins. These results suggested that no proteins were co-immunoprecipitated directly by 3-17-linked protein G-sepharose beads or in association with baculoviral proteins. This indicates that the proteins that were co-immunoprecipitated represented the results of interactions between the recombinant FeLV surface glycoproteins and the (labelled) cell surface proteins. No difference was observed between those proteins co-immunoprecipitated with Bgp70-A, Bgp70-C or Bgp70-215. A common species was observed in all the cell lines at 100 kDa. A species that was 70 kDa in size was noted in lysates prepared from HuT 78 cells but not in lysates prepared from other cell lines. This result contrasts markedly with those of other workers (Ghosh *et al.*, 1992). The differences in intensity that were observed in the various tracks were assumed to be due to normal experimental variation. As different species of protein were co-immunoprecipitated from all four cell lines the possibility that free ^{125}I or the iodination reaction mix had resulted in the labelling of the recombinant proteins was not addressed.

In a separate control experiment the ability of the Bgp100 protein to co-immunoprecipitate proteins from the labelled lysates was investigated. To perform the precipitations the monoclonal antibody VPG 71.2 was first coupled to protein G-sepharose beads as described above. Aliquots of 125 µl of the labelled cell lysates were incubated with 12.5 µg aliquots of Bgp70-A and Bgp100 for 90 minutes. After this time the mixtures were cross-linked and then co-immunoprecipitated with 10 µl of VPG 71.2-linked protein G-sepharose beads as before. The beads were analysed by SDS-PAGE and then an autoradiograph was prepared from the gel as described above over a period of 21 days. A photograph of the autoradiograph is shown in Figure 6.5.

Figure 6.5 demonstrates that no proteins were specifically co-immunoprecipitated

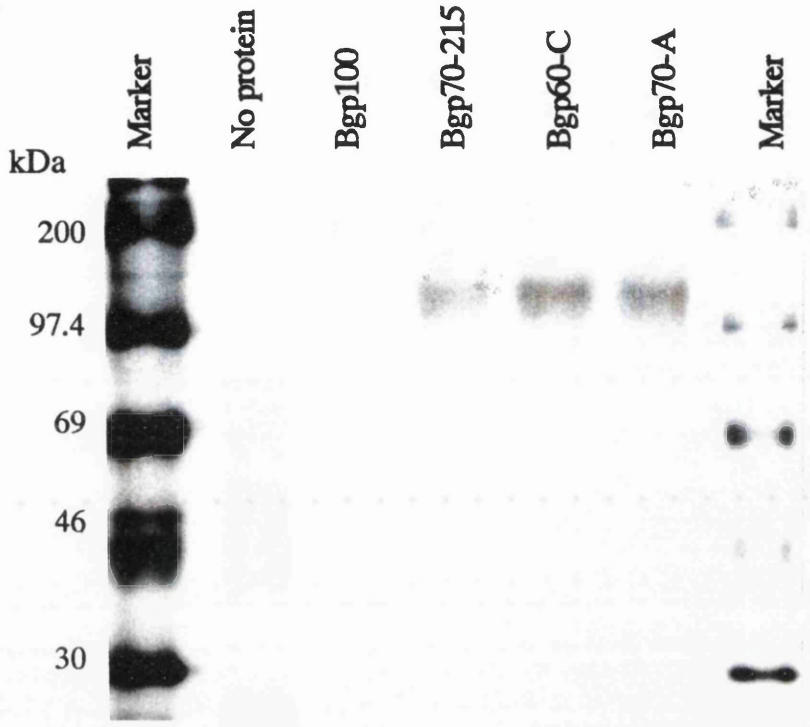
Figure 6.4 Co-immunoprecipitation of SU binding proteins with 3-17



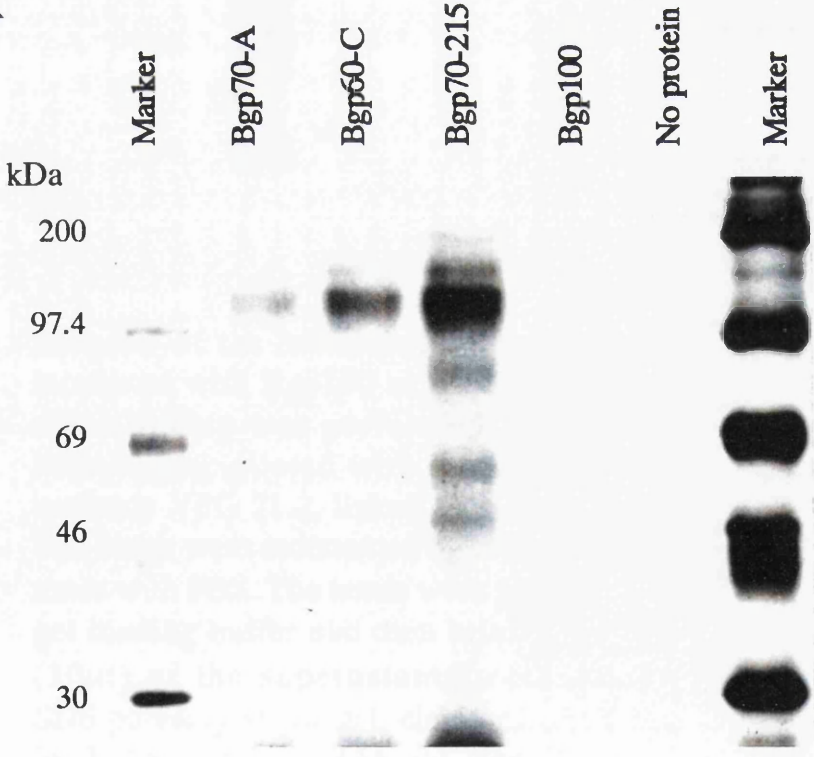
1 x 10^7 cells were surface labelled and then lysed with 0.5% NP40 in TBS. Non-specific binding was removed by incubation with Sepharose G25 (Pharmacia). Volumes of 250 µl of the supernatant were then incubated at room temperature for 90 minutes with 50 µl of the recombinant proteins. Chemical cross-linking was performed with the reducible crosslinker, DSP. Co-immunoprecipitation was performed with 3-17-linked protein G-sepharose beads. The beads were sedimented by centrifugation, washed 3 times with PBS and then were boiled in 30 µl of SDS gel loading buffer. After centrifugation, aliquots (10 µl) of the supernatants were loaded onto a 10% SDS-polyacrylamide gel and electrophoresis performed at 50 V for 2.5 hours. The gel was then fixed, dried and autoradiographed. The markers used were 14-C Rainbow markers (Amersham International).

Figure 6.4 (continued) Co-immunoprecipitation of SU binding proteins with 3-17

C) AH927

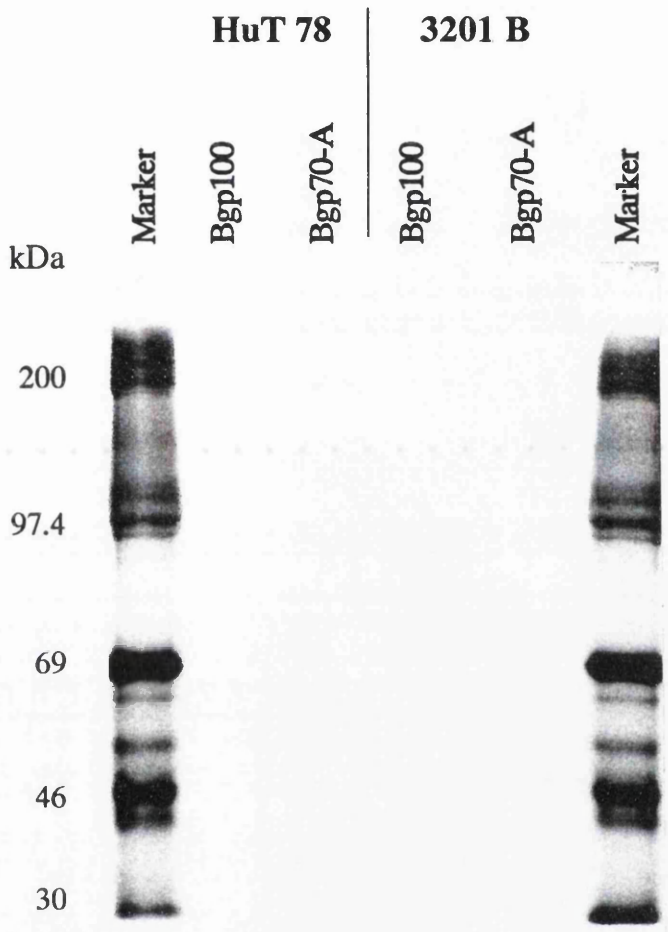


D) MDCK



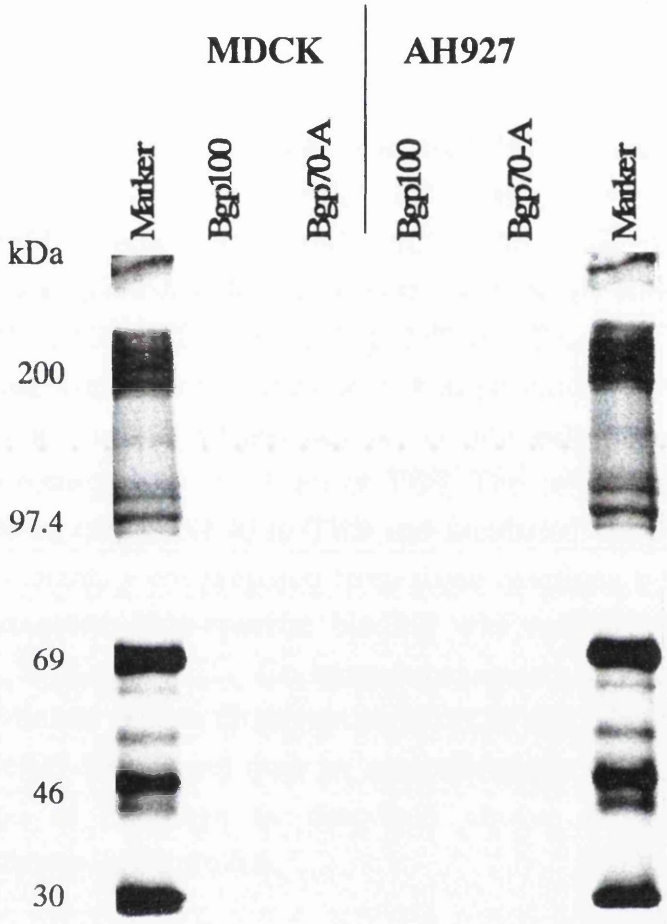
(For legend see previous page)

Figure 6.5 Co-immunoprecipitation of SU binding proteins with VPG 71.2



Aliquots of the radiolabelled lysate supernatants were incubated with Bgp100 or Bgp70-A as before. Chemical cross-linking was performed, then the proteins were immunoprecipitated with the anti-FIV SU monoclonal antibody VPG 71.2, linked to protein G-sepharose beads. The beads were sedimented by centrifugation and washed 3 times with PBS. The beads were then boiled in 30µl of SDS gel loading buffer and then briefly centrifuged. Aliquots (10µl) of the supernatants were loaded onto a 10% SDS-polyacrylamide gel; electrophoresis was performed at 50 V for 3 hours. The gel was then fixed, dried and autoradiographed. The markers used were 14-C Rainbow markers (Amersham International).

Figure 6.5 (continued) Co-immunoprecipitation of SU binding proteins with VPG 71.2



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with Bgp100 or Bgp70-A using the monoclonal antibody VPG 71.2. This result suggests a confirmation of the specificity of the co-immunoprecipitations shown in Figure 6.4.

(b) Whole cells

In an assay similar to that previously described for identifying FeLV cellular receptors (Ghosh *et al.,* 1992), surface iodinated HuT 78 and 3201 cells were prepared as detailed above and, after the final PBS wash, were resuspended in 1 ml of PBS with 50 mM glucose and 1 mM $CaCl_2$. The suspension was split into aliquots of 300 µl and incubated for 30 minutes with 50 µl aliquots of Bgp70-A, Bgp70-C Bgp70-215 or Bgp100 on a rotating platform. Chemical cross-linking was performed by adding $MgCl_2$ and DSP as described previously. The reactions were then incubated for a further 30 minutes before the cells were precipitated by centrifugation and resuspended in 0.9 ml of TBS. The cells were then lysed with the addition of 100 µl of 5% NP40 in TBS and incubated for 5 minutes at room temperature. Supernatants were prepared from these reactions by centrifugation at 6500 rpm for 5 minutes. Non-specific binding was reduced by incubating the supernatants with Sephadex G25. Co-immunoprecipitation was then performed with 10 µl of 3-17-linked protein G-sepharose beads as described above. The beads were analysed by SDS-PAGE and then an autoradiograph was prepared from the gel over a period of 10 days as described above. A photograph of the autoradiograph is shown in Figure 6.6.

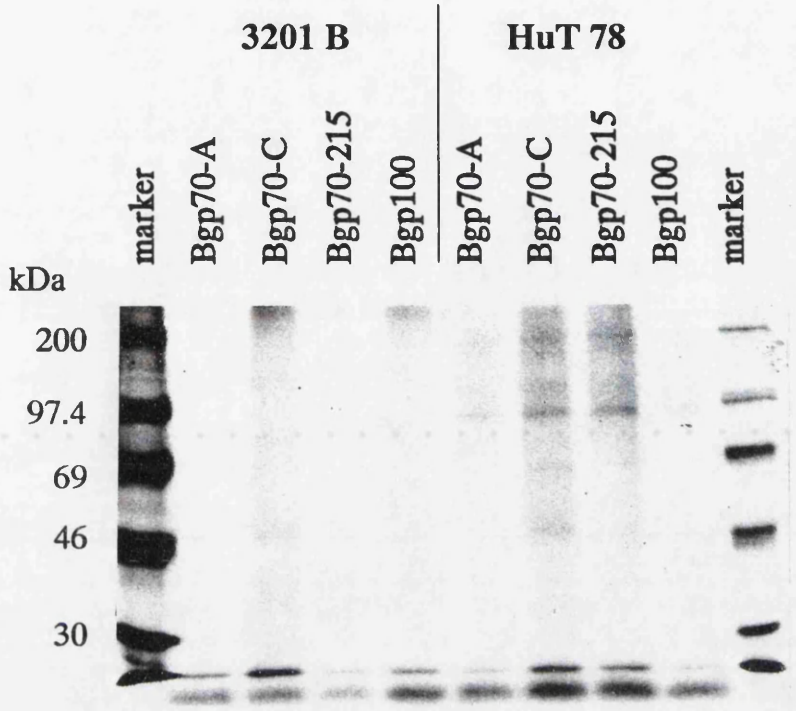
Figure 6.6 demonstrates that few protein species could be detected by this technique. The species that were detectable in the Hut 78 cell line were similar in size to those co-immunoprecipitated from the cell lysates. This suggested that this technique was less efficient than using cell lysates. From the result obtained with the control without protein in Figure 6.4, it was decided not to include a similar control for this experiment.

6.3 Discussion

This chapter describes the use of the recombinant FeLV surface glycoproteins to block the infection by FeLV of susceptible cells. The results from these experiments suggested that the proteins could be used as ligands with which to define the viral cell surface receptors (VCSRs) for FeLV.

The recombinant proteins interfered with the infection of susceptible cells in a subgroup specific manner. A chimeric protein containing the VR 1 region of FeLV-C in an FeLV-A backbone (Bgp70-215) blocked the infection of FeLV-C but not FeLV-A. This result corroborated data demonstrating that the VR 1 region of FeLV

Figure 6.6 Co- immunoprecipitation of SU binding proteins from whole cells



1 x 10^7 cells were surface labelled as before and were then incubated with the recombinant proteins for 30 minutes. Chemical cross-linking was performed with DSP and then the cells were lysed with 0.5% NP40 in TBS. The lysates were cleared by centrifugation and non-specific binding was removed by incubation of the supernatant for 1 hour with Sepharose G25 (Pharmacia). To co-immunoprecipitate putative receptor / ligand complexes an aliquot of 10 µl of 3-17-linked protein G-sepharose beads was added for 1 hour. The beads were then sedimented by centrifugation and washed 3 times with PBS. The beads were boiled in 30 µl of SDS gel loading buffer and then briefly centrifuged. Aliquots (10 µl) of the supernatants were loaded onto a 10% SDS-polyacrylamide gel and electrophoresis performed at 50 V for 2.5 hours. The gel was then fixed, dried and autoradiographed.
The markers used were 14-C Rainbow markers (Amersham).

determines the A/C subgroup phenotype (Rigby, 1989). As the existence of three FeLV subgroups has been taken as evidence of three FeLV receptors (Sarma and Log, 1973; Riedel *et al.,* 1986; Rigby *et al.,* 1992; Brojatsch *et al.,* 1992), it follows that the VR1 region represents at least part of a putative receptor binding site (RBS) for FeLV. The recombinant proteins therefore contain a receptor binding site (RBS) which functions in a similar fashion to the native RBS.

The chimeric Bgp70-215 protein functioned in a similar fashion to Bgp70-A in neutralisation inhibition assays (see section 5.2.3). These results demonstrated that, in the recombinant chimeric protein, the major neutralisation epitopes were separate from the subgroup determining region. This demonstration mirrored observations made from studies on native FeLVs (Russell and Jarrett, 1978b; Rigby, 1989) and served to emphasise the functional similarity of the recombinant and wild type proteins.

FeLV infection could be completely inhibited by increasing the quantity of the recombinant proteins. This result demonstrates that exogenous blockade can be as effective as the endogenous blockage observed during superinfection interference assays. Further work defining the precise qualities of this blockade may help to determine whether the phenomenon of superinfection interference is mediated entirely at the cell surface or partly post-binding. It has been suggested that FeLV receptors are expressed, albeit at a lower level, in infected cells (Ghosh *et al.,* 1992).

The subgroup specific interference that was observed is best explained in terms of receptor blockade. However, no conclusive evidence is presented that this is indeed the case. Confirmation of receptor blockade would require competitive binding studies involving preparations of highly purified recombinant and native proteins, one of which had been specifically labelled. Interaction could occur between the recombinant proteins and the native, virion-associated, surface glycoprotein, such that the virus was inactivated. However, it is suggested that any such process is unlikely to be subgroup specific. For example, the formation of hetero-oligomers composed of the recombinant and native proteins could result in inactivation. However, it is known that the formation of native SU oligomers is dependent on a portion of the amino terminus of the transmembrane protein, TM (Einfeld and Hunter, 1988). In FeLV the predicted amino acid sequences of this segment are constant between the three subgroups. Therefore, the formation of such hetero-oligomers would be, almost certainly, subgroup independent.

The ability of recombinant, RBS-containing, proteins to block the infection of a susceptible cell line has previously been reported for Epstein-Barr virus (Tanner *et*

al., 1988). The infection of susceptible cell lines has also been blocked by synthetic peptides corresponding to putative RBSs. For example, the cellular receptor of vaccinia virus was identified as the epidermal growth factor receptor by the ability of a synthetic peptide, corresponding to a region of the envelope glycoprotein that was homologous with epidermal growth factor, to block plaque formation (Eppstein *et al.,* 1985). These workers also demonstrated that an additional decrease in the number of plaques was obtained when the peptide was included after, as well as during, the virus adsorption phase. A similar observation was made in the preliminary experiments used to define the conditions for the infection interference assays. Further experiments should be performed to optimise the assay and investigate this phenomenon.

It would be interesting to repeat the FeLV-C infection interference experiment in the presence of FeLV-A neutralising serum and Bgp70-215. This would determine if FeLV-A neutralising antibodies acted by interfering with receptor binding. Such antibodies would not have an effect on FeLV-C, but if they induced conformational changes or steric hindrance of the receptor binding properties of Bgp70-215 then the infection interference properties of this recombinant protein would be reduced. It would not be possible to use Bgp70-A for this experiment as it does not interfere with FeLV-C. Similarly, it would not be possible to use FeLV-A in this experiment as the neutralising antibodies would also act on the virus. Such studies would help to define the action of neutralising antibodies in the FeLV system. It has been demonstrated that the antibody class can influence the mechanism of action (Taylor and Dimmock, 1985). In that study secretory IgA was found to block receptor binding, but neutralising IgG and monomeric IgA molecules did not prevent receptor binding. It would probably be technically simpler to perform the above experiment than to measure directly the binding of the chimeric virus in the presence of FeLV-A neutralising serum.

This is the first report of recombinant proteins that are capable of directly interfering with FeLV infection. The availability of such reagents may facilitate the identification of the FeLV cellular receptors. The ability of the recombinant proteins to accurately mimic the subgroup phenotype of the native gp70 was crucial to the relevance of the subsequent co-immunoprecipitation experiments. These studies were performed with AH927 and 3201 B cells, which are susceptible to FeLV-A (Rasheed and Gardner, 1980; Rojko *et al.,* 1991) together with MDCK and HuT 78 cells, which are not thought to be susceptible to FeLV-A (Jarrett *et al.,* 1972; Sarma *et al.,* 1975). AH927 and 3201 B cells are also susceptible to FeLV-C (Rasheed and Gardner, 1980; Rojko *et al.,* 1991). The ability of MDCK and HuT 78 cells to support the growth of FeLV-C is unknown and was not investigated.

The co-immunoprecipitation studies demonstrated that a number of cell surface proteins on both susceptible and non-susceptible cell lines interact with recombinant FeLV surface glycoproteins. The lack of subgroup specificity in the co-immunoprecipitation reactions contrasted markedly with the subgroup specificity of the infection interference assays. The number of proteins that were co-immunoprecipitated strongly suggests that the recombinant FeLV surface glycoproteins cannot be used in this manner to directly identify FeLV cellular receptors. These results are at variance with the results of Ghosh *et al.*, (1992), which indicated that isolated native gp70 co-immunoprecipitated one protein species from 3201B cells and none from HuT 78 cells. A major drawback of that study was the failure to demonstrate that the preparation of SU was capable of specifically blocking virus entry.

Co-immunoprecipitation of cross-linked protein complexes is, notwithstanding the above caveats, a specific method of identifying stable interactions between proteins (Brenner *et al.*, 1985). From the negative results of the co-immunoprecipitation studies with VPG 71.2 it is probable that the cell surface proteins co-immunoprecipitated in the set of reactions illustrated in Figure 6.4 do represent the results of non-random interactions with the recombinant baculoviral proteins. However, the efficiency of the protein G coupling was not assessed for either 3-17 or VPG 71.2 and the relative binding abilities of the immobilised monoclonal antibodies were not compared. Furthermore, a conformational change of Bgp100 could be induced by binding to a cell surface protein such that VPG 71.2 could no longer immunoprecipitate Bgp100. Therefore, it is possible that this control experiment does not permit an assessment to be made of the specificity of the main set of reactions. This indicates that there is a slight possibility that some or all of these cell surface proteins are the results of random interactions and are not involved in virus binding or entry. Repeating this experiment with an FIV susceptible cell line with a preparation of recombinant FIV SU that was known to block infection would help to unravel this conundrum. Another modification that could be made in future experiments is to alter both the concentration and the composition of the lysis buffer in which the reactions were carried out. This could help to increase further the specificity of the reactions.

It is possible that the actual initial attachment of the virus to the cell is not mediated by the VCSR/RBS interaction, but rather by other, less specific, transient, protein-protein interactions. Such interactions, though not random, need not be specific to susceptible cells and could involve several proteins; unless the RBS subsequently recruits a VCSR, the virus will still fail to penetrate the cell. Therefore, another interpretation of the results could be that the experiments had identified several cell

surface proteins involved in this initial phase. This explanation accounts for the large number of protein species that were specifically co-immunoprecipitated.

The cell surface proteins involved in these transient non-specific protein-protein interactions might also interact in a similar fashion with other viral proteins. The use of Bgp100 in this chapter was an attempt to control for this variable. As it was not possible to prove that Bgp100 did not bind to any of the cell lines, the interpretation of the results as suggesting that the Bgp70 proteins bound to cell surface proteins that were involved in the initial transient phase is still valid. If the results of these experiments are interpreted in either of the above fashions, then they would suggest that to use co-immunoprecipitation with recombinant viral proteins to identify that specific entity which serves as the VCSR is fraught with the potential for non-specific interaction. By implication, experiments involving preparations of native glycoprotein, especially those which have not been demonstrated to block viral infection (Ghosh *et al.,* 1992), are also open to over-interpretation. It should be noted that the conditions used in the co-immunoprecipitation experiments described in this chapter resemble those used by Ghosh *et al.* to identify a 70 kDa protein as a putative FeLV receptor.

It is interesting that, prior to the identification of the ecotropic MuLV receptor as the basic amino acid transporter, y^+ (Albritton *et al.,* 1989; Kim *et al.,* 1991; Wang *et al.,* 1991), several attempts had been made to identify the receptor using co-immunoprecipitation techniques with ^{125}I surface labelled cells (Robinson *et al.,* 1980; Schaffar-Deshayes *et al.,* 1981). The earlier study demonstrated a 14 kDa protein on the surface of leucocytes that bound to MuLV gp85 complexes. The later study demonstrated a 190 kDa protein on the surface of thymus cells that bound to MuLV gp71. The predicted molecular mass of the MuLV receptor is 67 kDa (Albritton *et al.,* 1989). These results also suggest that using co-immunoprecipitation to identify viral cellular receptors may be an unreliable technique.

The experiments described in this chapter and elsewhere (Reinhart *et al.,* 1993) do suggest that superinfection interference and virus binding may be more complicated than previously assumed. Superinfection interference may be due to blockade of (subgroup specific) cell proteins that are required for virus entry, but not for primary virus binding (Reinhart *et al.,* 1993). Further studies on the ability of non-susceptible and susceptible cells to bind FeLV, or its recombinant surface glycoprotein, are required.

Many other viral cellular receptors have been identified using monoclonal antibodies which block virus infection (Fingeroth *et al.,* 1984; Dalgleish *et al.,*

1984; Greve *et al.,* 1989; Staunton *et al.,* 1989; Bergelson *et al.,* 1992). These monoclonal antibodies are usually raised against susceptible cells. As a result of the large number of proteins in these preparations, many hybridomas may have to be screened before such a monoclonal antibody is identified. As it is likely that the recombinant proteins do bind to the FeLV receptors (as well as other proteins), it may be possible by affinity purification using the recombinant proteins to decrease the number of contaminating proteins in the susceptible cell preparation used to immunise the mice. This might decrease the number of hybridomas that subsequently have to be screened.

The receptors for gibbon ape leukaemia virus and ecotropic murine leukaemia virus were identified by transfection of cDNA into non-permissive cells, followed by probing with recombinant retroviruses expressing drug resistance genes (Albritton *et al.,* 1989; O'Hara *et al.,* 1990). It is suggested that the identification of the receptors for FeLV may require this type of approach.

Chapter 7. Vaccination Studies

7.1 Introduction

This chapter describes a trial of the recombinant FeLV-A surface glycoprotein, Bgp70-A, as a candidate FeLV vaccine. In previous chapters it has been demonstrated that this protein is recognised by the sera of cats which have recovered from FeLV infection (see sections 5.2.1 and 5.2.2). The protein also inhibits the action of neutralising antibodies *in vitro* (see section 5.2.3). These results suggested that the protein might be able to generate neutralising antibodies *in vivo* and thereby induce protection against FeLV challenge.

No commercially available FeLV vaccine has been demonstrated to elicit neutralising antibodies prior to challenge with FeLV. One experimental vaccine containing native gp70 incorporated into ISCOMs has been demonstrated to elicit pre-challenge neutralising antibodies (Osterhaus *et al.,* 1985; Osterhaus *et al.,* 1989). The development of a vaccine that was commercially viable and induced prechallenge neutralising antibodies would be of considerable clinical benefit. The presence of neutralising antibodies, even at very low titres, is thought to confer protection against subsequent viral challenge (Jarrett *et al.,* 1977; Russell and Jarrett, 1978b; deNoronha *et al.,* 1978). A vaccine was prepared from the recombinant protein and included as part of a larger trial of several other experimental FeLV vaccines.

Recombinant soluble proteins are often weak immunogens (Byars and Allison, 1988; Reid, 1992; Gupta *et al.,* 1993). It was decided that a potent adjuvant should be used in the vaccine trail. Quil A is purified by aqueous extraction from saponin, which is a mixture of glycosides obtained from the bark of a South American tree, *Quilaja saponaria* Molina. It has been demonstrated to have adjuvanting properties and is used in the formation of ISCOMs (Smith *et al.,* 1981; Morein *et al.,* 1984; Bomford *et al.,* 1992). QS-21 is a component of Quil A, purified by reverse phase chromatography, which has been demonstrated to have minimal toxicity, whilst retaining the adjuvant properties of Quil A (Kensil *et al.,* 1991). QS-21 is used as an adjuvant in Leucogen (Virbac), which is one of the FeLV vaccines that are commercially available in the UK. Quil A is used as an adjuvant in the other commercially available FeLV vaccine, Leukocell 2 (SmithKline Beecham Animal Health).

7.2 Results

7.2.1 Experimental Animals

The specific pathogen free (SPF) cats used in this experiment were obtained from a commercial source when they were 7 weeks old and were kept in a barrier maintained cattery at Glasgow University. The cats were fed on a diet of Whiskas (Pedigree Petfoods) and heat sterilised milk.

Throughout the experiment the welfare of the animals was carefully monitored by trained technical staff under the supervision of veterinary surgeons. Experimental procedures were carried out within the terms of the Animals (Scientific Procedures) Act, 1986. At the end of the experiment (week 19 - see below) all the cats were killed using pentobarbitone, which was administered intravenously.

On arrival at the cattery each cat was injected subcutaneously with 0.2 ml of a long acting preparation of amoxycillin (Clamoxyl LA, Smithkline Beecham Animal Health) and assigned, at random, into one of three groups. Antibiotic cover was provided as previous experience had indicated that mild outbreaks of bacterial respiratory disease often accompanied transportation and rehousing of the cats. Group 1 contained five cats that were subsequently vaccinated with the experimental vaccine and were numbered 171 to 175. Group 2 contained five cats that were subsequently vaccinated with a commercial FeLV vaccine (Leucogen; Virbac) and were numbered 181 to 185. Group 3 contained five cats that were not vaccinated and therefore served as the control group. These cats were numbered 186 to 190.

7.2.2 Preparation of Experimental Vaccine

The recombinant protein vaccine was prepared using a stock of Bgp70-A protein that contained 0.3 mg/ml of total protein. A 4.0 ml volume of the recombinant protein was formulated into a vaccine with the addition of 0.3 ml of TBS, 0.9 ml of a 3% suspension of aluminium hydroxide (Superfos) and 265 µl of a 1 mg/ml preparation of Quil A (Superfos; batch number 01046, expiry date February 2003). The vaccine was prepared on the day of use and stored on ice.

7.2.3 Vaccination Regime

A subcutaneous injection of 1 ml of the experimental vaccine was given to each cat in Group 1 on two occasions, with an interval of 3 weeks. The cats in Group 2 were vaccinated at the same time with Leucogen (batch number, 809857/2 K075: expiry date, January 1995). The cats in Group 3 were not vaccinated. After each vaccination the cats were closely monitored for signs of illness. The first

vaccinations were given when the cats were 8 weeks old; this time point was taken as week 0 of the experiment. For a period of 24 hours after the initial vaccination all the cats in Groups 1 and 2 were lethargic. All the cats recovered without treatment. No adverse reactions were seen after the second vaccine dose.

Five weeks after the first vaccination all the cats were challenged by oronasal inoculation of 1 ml of culture medium containing 1 x 10^6 pfu of FeLV-A/Glasgow-1. This was performed by Professor O. Jarrett by introducing a 0.5 ml aliquot into the cat's oropharynx and 0.25 ml aliquots into each nasal chamber.

Heparinised blood samples were obtained from the cats by jugular venepuncture at weeks 0, 2, 5, 8, 11, 14 and 19 of the experiment. The plasma was prepared from the blood samples by centrifugation at 2000 rpm for 5 minutes in a GPR centrifuge and was then frozen in aliquots at -70°C until required.

7.2.4 Analysis of Responses to Vaccination

(1) FeLV assays

Viraemia was assessed by the staff of the Feline Virus Unit using a p27 competition ELISA and by virus isolation. The p27 ELISA was performed using p27 antigen coated wells (Inochem). An 80 µl aliquot of an anti-p27 monoclonal antibody-horseradish peroxidase conjugate was added to each well, followed by a 15 µl aliquot of the test serum. Positive and negative control sera were included in each set of reactions. The wells were incubated for 1 hour at room temperature and then were washed with a 150 mM NaCl, 0.05% Tween 20 solution. During this time the substrate solution was prepared using a commercial kit (TMB microwell peroxidase substrate system; Kirkegaard and Perry Laboratories) by mixing equal volumes of 3,3',5,5' tetramethylbenzidine (TMB) at a concentration of 0.4 g/l in an organic solvent and 0.02% H_2O_2 in a citric acid buffer. After washing the wells were blotted dry and then a 100 µl aliquot of the freshly prepared TMB substrate solution was added to each well. The reactions were incubated for a further 15 minutes. The results were read at 650nm using an EL312 Bio-Kinetics ELISA plate reader (Bio-Tek Instruments).

Viral isolation was performed by seeding QN10S cells in 12-well tissue culture plates at a density of 4 x 10^4 cells per well with 1.0 ml culture fluid containing 4 µg/ml of polybrene. The next day 200 µl of the plasma was inoculated into the culture fluid and the cells incubated for 2 hours. The inoculum was then removed and the cells were incubated for a further 6 days with fresh culture fluid. After this time the cells were examined for foci of transformation. The cells in those wells without evidence of transformation were routinely subcultured for a further 4 days

before final scoring.

(2) Anti-gp70 antibody assays

The presence of anti-gp70 antibodies in the plasma of the cats was assessed by virus neutralisation and western blotting assays.

The presence of neutralising antibodies was assessed by the staff of the Feline Virus Unit using a method similar to that described previously for the neutralisation inhibition assay (see section 5.2.3). To perform these assays, QN10S cells were seeded in 12-well tissue culture plates at a density of 4 x 10^4 cells per well with 1.0 ml culture fluid containing 4 μg/ml of polybrene. The next day twofold dilutions of 50 μl of the heat inactivated plasma were made in 96-well round bottomed microtitre trays. A stock of FeLV-A was diluted to 4 x 10^2 pfu/ml and then 50 μl aliquots were added to the plasma dilutions. The microtitre tray was incubated in a humidified incubator for 6 hours. Samples of 25 μl were then added to the culture fluid of the QN10S cells. After 2 hours the culture fluid was removed and the cells were incubated with fresh medium for a further 6 days. After this time the cells were examined for foci of transformation. The end point of assay was taken as that dilution of plasma which produced a 75% reduction in the number of plaques compared to SPF control serum. Recovered cat serum P11 was used as a positive control in these assays.

The western blot analyses of plasma derived from the cats were performed by Dr. J. Christie and Mr. M. Golder (Feline Virus Unit). A nitrocellulose membrane was prepared from a 15% SDS-PAGE gel using FeLV derived from F422 culture fluid. An aliquot of 100 μl of the vaccine protein and 30 μl of concentrated F422 culture fluid were electrophoresed at 100 V until the sample dye reached the bottom of the gel. The proteins were then transferred to the membranes. The membranes were incubated overnight with blocking solution (see section 2.1.3) supplemented with 20% goat serum. The next day they were washed 3 times with PBS-Tween (see section 2.1.3). The membranes were stored in a moisture free environment in an airtight plastic bag at -20^{0}C until required.

The plasmas were preincubated overnight with blocking solution to which had been added 20% goat serum, 0.2 % (v/v) 0.5 M EDTA and 0.5% (v/v) Tween 20 ('modified blocking solution'). Supernatants were prepared by centrifugation in a benchtop benchtop centrifuge at 6000 rpm for 5 minutes. The nitrocellulose membranes were sliced into 2 mm wide strips which were then placed in multiwell trays. The blots were incubated with the supernatants for 2 hours. After washing with PBS-Tween, the blots were incubated with biotinylated protein A, diluted 1 in 500 with modified blocking solution, and then, after washing with PBS-Tween,

with streptavidin-alkaline phosphatase conjugate, which was also diluted 1 in 500 with modified blocking solution. The western blot strips were developed with BCIP/NBT (as described in section 2.3.5). The blots were scored empirically by comparison with controls performed with recovered cat serum P11. A slight variation in sensitivity was noted between the different blots that were prepared for each set of samples.

The results of these tests are given in Tables 7.1 and 7.2. None of the cats were viraemic or p27 antigenaemic prior to challenge. No cat prior to challenge developed virus neutralising antibodies. Only the cats vaccinated with Leucogen developed anti-gp70 antibodies on western blot analysis prior to challenge. Following challenge with FeLV all the unvaccinated cats became viraemic and antigenaemic. One cat, F187, later developed neutralising antibodies and became aviraemic, though it was still p27 antigenaemic at the end of the trial. It is probable that, had the experiment been continued, this cat would have fully recovered from the infection. In this group therefore four of the cats became persistently viraemic and the other cat became transiently viraemic. Small quantities of anti-gp70 antibodies were detectable in some of the other control cats by the end of the trial. This is common in persistently viraemic cats (Lutz *et al.,* 1980).

Four of the cats vaccinated with Leucogen became persistently viraemic. The remaining cat in Group 2, F183, was positive on the p27 ELISA during week 8, but no infectious virus was ever isolated and the p27 antigenaemia regressed in subsequent samples. This result suggested that a low level of FeLV replication had occurred in this cat. This cat developed neutralising antibodies by the end of the trial.

In the group of cats vaccinated with the recombinant protein preparation (Group 1) two out of the five cats were p27 and virus negative during week 8, but subsequently one of these (number 175) became persistently viraemic. Cat number 173 never became antigenaemic or viraemic. Only cat number 173 produced anti-gp70 antibodies that were readily detectable using western blot analysis: it also developed virus neutralising antibodies 9 weeks after challenge. The other cats in this group produced anti-gp70 antibodies that were only barely detectable on western blot.

These results suggest that neither of the vaccines were effective at preventing the development of persistent viraemia in the cats.

(23 Analysis of response to vaccine protein

A series of western blots were performed to further analyse the antibody responses

Table 7.1 Assays for FeLV in vaccinated cats

GROUP: Vaccine	Week	0		5		8		11		14		19	
	Stage	Pre-vaccination		Pre-challenge		3 weeks post-challenge		6 weeks post-challenge		9 weeks post-challenge		14 weeks post-challenge	
	Animal	p27 ELISA	VI	p27 ELISA	VI	p27 ELISA	VI	p27 ELISA	VI	p27 ELISA	VI	p27 ELISA	VI
1: Bgp70-A	F171	-	-	-	-	+	+	+	+	+	+	+	+
	F172	-	-	-	-	+/-	+	+	+	+	+	+	+
	F173	-	-	-	-	-	-	-	-	-	-	-	-
	F174	-	-	-	-	+	+	+	+	+	+	+	+
	F175	-	-	-	-	-	-	+	+	+	+	+	+
2: Leucogen	F181	-	-	-	-	+	+	+	+	+	+	+	+
	F182	-	-	-	-	+	+	+	+	+	+	+	+
	F183	-	-	-	-	+	-	-	-	-	-	-	-
	F184	-	-	-	-	+	+	+	+	+	+	+	+
	F185	-	-	-	-	+	+	+	+	+	+	+	+
3: Unvaccinated	F186	-	-	-	-	+	+	+	+	+	+	+	+
	F187	-	-	-	-	+	+	+	+	+	+	+	-
	F188	-	-	-	-	+	+	+	+	+	+	+	+
	F189	-	-	-	-	+	+	+	+	+	+	+	+
	F190	-	-	-	-	+	+	+	+	+	+	+	+

This table shows the results obtained from assays for the presence of FeLV in the cats' plasma samples during the course of the experiment. p27 antigenaemia was assessed using a p27 competition ELISA. Virus isolation (VI) was performed by incubation of a 200 µl aliquot of plasma with QN10S cells for 2 hours, followed by incubation of the cells in fresh medium for 6 days. A result was only scored negative if no evidence of transformation was present following subculture for a further 4 days.

Table 7.2 Assays for anti-FeLV gp70 antibodies in vaccinated cats

	Week	0		5		8		11		14		19	
	Stage	Pre-vaccination		Pre-challenge		3 weeks post-challenge		6 weeks post-challenge		9 weeks post-challenge		14 weeks post-challenge	
GROUP:Vaccine	Animal	WB	VN	WB	VN	WB	VN	WB	VN	WB	VN	WB	VN
1: Bgp70-A	F171	-	-	-	-	ND	-	-	-	+	-	-	-
	F172	-	-	-	-	ND	-	-	-	+	-	+	-
	F173	-	-	-	-	ND	-	+	-	+++	>16	++	128
	F174	-	-	-	-	ND	-	-	-	+	-	+	-
	F175	-	-	-	-	ND	-	-	-	+	-	-	-
2: Leucogen	F181	-	-	+	-	ND	-	ND	-	+++	-	++	-
	F182	-	-	+	-	ND	-	ND	-	+++	-	++	-
	F183	-	-	+	-	ND	-	ND	-	+++	-	++	32
	F184	-	-	+	-	ND	-	ND	-	+++	-	++	-
	F185	-	-	+	-	ND	-	ND	-	+++	-	++	-
3: Unvaccinated	F186	-	-	-	-	ND	-	+	-	+/-	-	-	-
	F187	-	-	-	-	ND	-	+	-	+	16	+	8
	F188	-	-	-	-	ND	-	+	-	+	-	+/-	-
	F189	-	-	-	-	ND	-	+	-	+	-	-	-
	F190	-	-	-	-	ND	-	+	-	+	-	-	-

This table shows the results obtained from western blot (WB) and virus neutralisation (VN) assays for the presence of anti-gp70 antibodies in the cats' plasma samples during the course of the experiment. WB assays were performed by incubating plasma samples diluted 1 in 10 with nitrocellulose membranes prepared using FeLV derived from F422 culture fluid and were scored relative to P11 serum. The titres of VN assays are shown: these assays were performed by incubation of a 25 µl sample of a neutralisation reaction, containing FeLV-A and dilutions of plasma, with QN10S cells for 2 hours, followed by incubation of the cells in fresh medium for 6 days. ND = not done.

of the experimental cats which were vaccinated with Bgp70-A. The cats' plasma samples were used to probe nitrocellulose membranes onto which had been blotted either the Bgp70-A vaccine protein, or FeLV proteins derived from F422 cell culture fluid. The membranes were prepared from two single well 5-20% gradient SDS-polyacrylamide gels. An aliquot of 100 µl of the vaccine protein and 30 µl of concentrated F422 culture fluid were electrophoresed at 100 V until the sample dye reached the bottom of the gel. The proteins were then transferred to the membranes. The membranes were incubated overnight with blocking solution (see section 2.1.3) supplemented with 20% goat serum. The next day they were washed 3 times with PBS-Tween (see section 2.1.3). The membranes were stored in a moisture free environment in an airtight plastic bag at -20°C until required.

To reduce non-specific activity, a preincubation solution was prepared by mixing equal volumes of concentrated supernatants harvested from wild type baculovirus infected, and mock infected, Sf9 cells (preparations C6 and C5 - see section 4.2.3). Aliquots of 10 µl of each cat's plasma from the 0, 5 and 11 week samples were incubated overnight at 4°C with 100 µl volumes of the preincubation solution. The next day each reaction was added to a 100 µl aliquot of blocking solution to which had been added 20% goat serum, 0.2 % (v/v) 0.5 M EDTA and 0.5% (v/v) Tween 20 ('modified blocking solution'). Each reaction was then centrifuged at 6000 rpm in a bench top benchtop centrifuge for 3 minutes and the supernatants were retained. The final concentration of the plasma was therefore 1 in 20.

The two nitrocellulose membranes were sliced into 2 mm wide strips which were then placed in multiwell trays. The strips were orientated by placing a diagonal cut at the top of each. The strips were washed twice with PBS-Tween and were then incubated with the preincubated supernatants. After 2 hours, the strips were washed with PBS-Tween (see section 2.1.3) for 30 minutes. Goat anti-cat IgG-alkaline phosphatase conjugate (Sera Lab), diluted 1 in 300 with modified blocking solution, was added to the strips in 100 µl aliquots for 1 hour.

Control sera from a recovered cat (P11), a SPF cat (Wendy) and a pooled preparation of recovered cat sera (PRCS) were prepared by adding 10 µl samples to 100 µl aliquots of the preincubation solution. To these preparations were added 100 µl volumes of modified blocking solution. The preparations were left for 15 minutes at room temperature and then centrifuged at 6000 rpm in a bench top benchtop centrifuge for 3 minutes. The nitrocellulose strips were incubated with 100 µl aliquots of the supernatants. After 2 hours, the strips were washed with PBS-Tween and then incubated with the conjugate preparation as above.

An ascites preparation of an anti-FeLV gp70 monoclonal antibody, 6-15, was

diluted 1 in 100 with modified blocking solution and used to probe a nitrocellulose strip from each of the membranes. In the wells containing these strips a goat anti-mouse IgG-alkaline phophatase conjugate (BioRad) was used as the second antibody. This conjugate was diluted 1 in 500 with modified blocking solution.

After incubation with the antibody conjugates, all the strips were washed three times with PBS-Tween for 1 hour, then with AP buffer for 5 minutes. The western blot strips were developed with BCIP and NBT in AP buffer (as described in section 2.3.5). Photographs of the western blots are shown in Figure 7.1. In this figure, the sera obtained at 0, 5 and 11 weeks are referred to as the prevaccination, prechallenge and 6 weeks post challenge (6 w.p.c.) samples respectively.

Photographs of some of the western blot analyses, performed by Dr. J. Christie and Mr. M. Golder (Feline Virus Unit), of samples derived from the cats vaccinated with Leucogen are shown for comparative purposes in Figure 7.2. The method is given above in section 8.2.4. The samples shown were derived from weeks 0, 5 and 14 of the experiment and are referred to as the prevaccination, prechallenge and 9 weeks post challenge (9 w.p.c.) respectively.

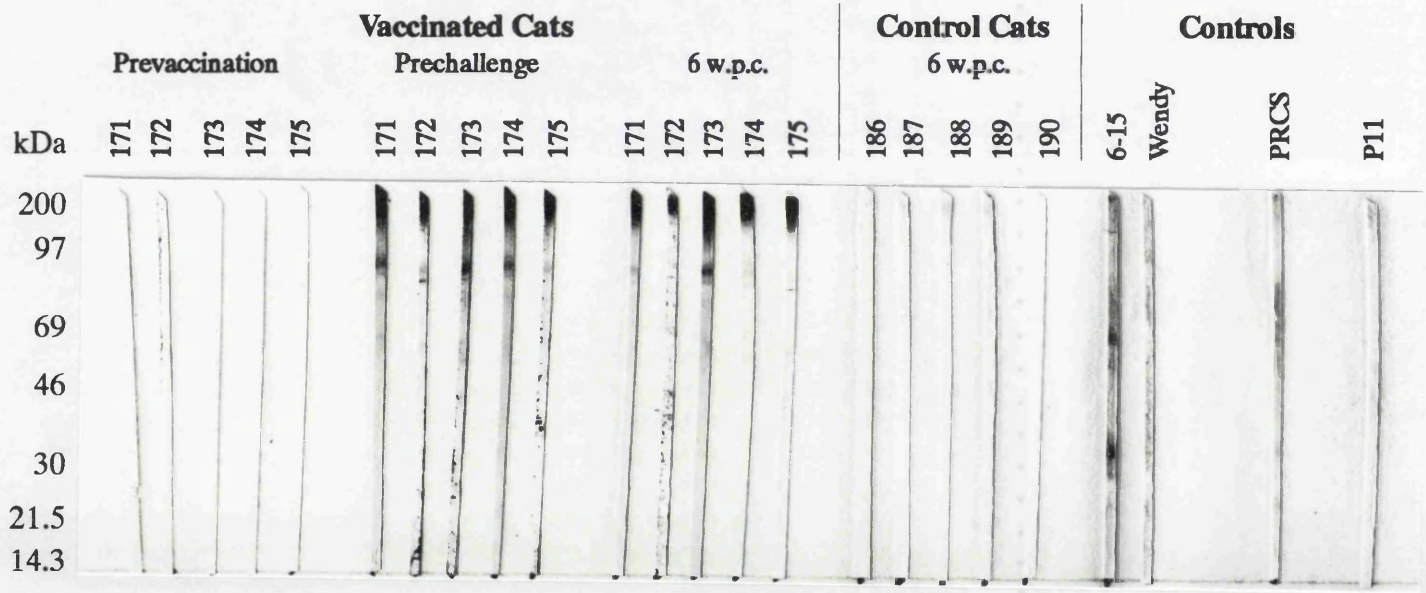
These photographs show that all of the Leucogen vaccinated cats developed anti-gp70 antibodies prior to challenge. In contrast, none of the cats vaccinated with the recombinant baculovirus derived material developed anti-gp70 antibodies prior to challenge. In accordance with the results obtained from the Feline Virus Unit, one cat (number 173) from Group 1 did develop anti-gp70 antibodies 6 weeks post infection. This was the same cat that did not develop viraemia. All of the cats in Group 1 developed specific responses to species of protein in the vaccine preparation, but did not develop responses to the species recognised by the 6-15 monoclonal antibody. No response was apparent to the presumed baculoviral gp64 protein. This indicated that the preincubation had removed non-specific activity. The results therefore suggest that the cats preferentially and specifically recognised a higher molecular weight species of the recombinant protein. These higher molecular weight forms were also apparent in western blots shown previously in Figure 5.1. None of the control cats developed titres of anti-gp70 antibodies as high as those of the Leucogen vaccinated cats.

7.3 Discussion

This chapter describes a vaccine trial involving the FeLV-A recombinant glycoprotein preparation, Bgp70-A. Only the FeLV-A recombinant glycoprotein was included in the experimental vaccine as it is this subgroup that is thought to be transmitted between cats in the natural situation (Jarrett *et al.,* 1978; Donahue *et*

Figure 7.1 Western blot analysis of sera from vaccination experiment

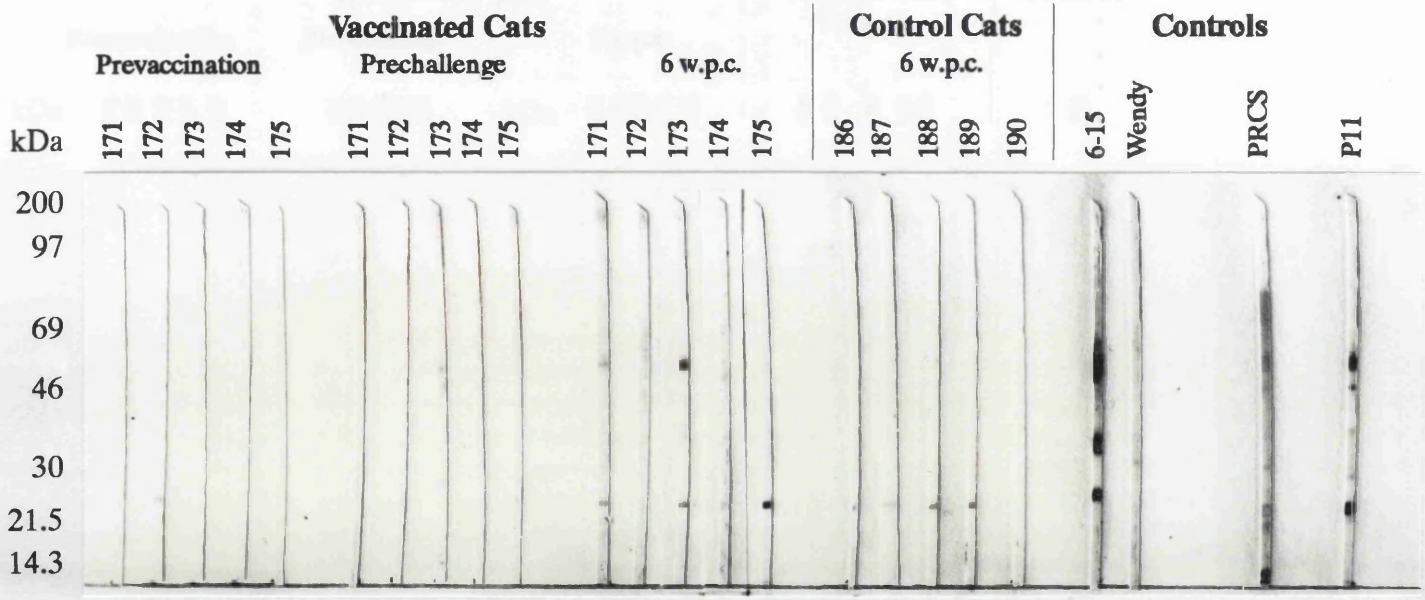
A) Recombinant Protein, Bgp70-A



This figure shows the western blot analyses of sera derived from the vaccinated and control cats taken at various stages during the course of the experiment. The blot was prepared from a single well 5-20% gradient SDS-polyacrylamide gel. A sample containing the recombinant protein preparation (Bgp70-A) was electrophoresed for 90 minutes at 100 V. Electroblotting was then performed and the blot stored at -20 C. Strips (2 mm wide) were cut from the blots and were then probed with 1 in 20 dilutions of sera from the vaccinated and control cats. After 2 hours the strips were washed with PBS-tween and then incubated with goat anti-cat IgG - alkaline phosphatase conjugate, diluted 1 in 300, for a further hour. The western blot strips were developed with NBT and BCIP in AP buffer. Control western blots were performed with the 6-15 monoclonal antibody, a pooled recovered cat serum (PRCS), a SPF cat serum (Wendy) and a recovered cat serum (P11). 6 w.p.c. = 6 weeks post challenge.

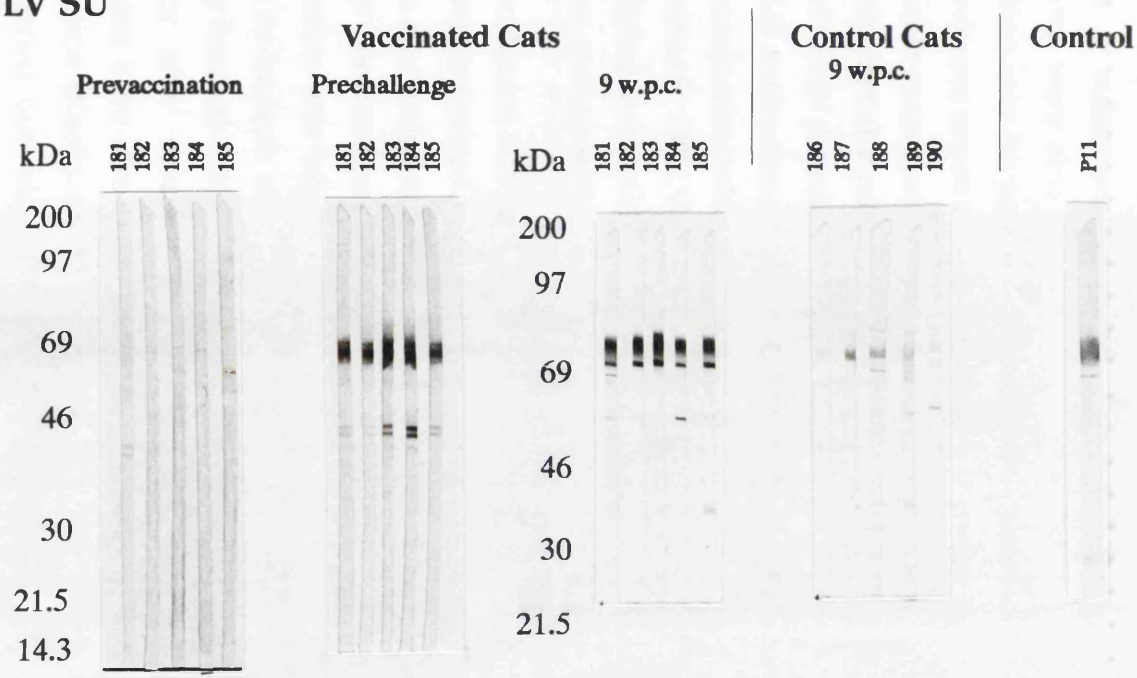
Figure 7.1 Western blot analysis of sera from vaccination experiment (continued)

B) Native FeLV SU



This figure shows the western blot analyses of sera derived from the vaccinated and control cats taken at various stages during the course of the experiment. The blot was prepared from a single well 5-20% gradient SDS-polyacrylamide gel. A sample containing native FeLV gp70 derived from the F422 cell line was electrophoresed for 90 minutes at 100 V. Electroblotting was then performed and the blot stored at -20 C. Strips (2 mm wide) were cut from the blots and were then probed with 1 in 20 dilutions of sera from the vaccinated and control cats. After 2 hours the strips were washed with PBS-tween and then incubated with goat anti-cat IgG - alkaline phosphatase conjugate, diluted 1 in 500, for a further hour. The immunoblot strips were developed with NBT and BCIP in AP buffer. Control western blots were performed with the 6-15 monoclonal antibody, a pooled recovered cat serum (PRCS), a SPF cat serum (Wendy) and a recovered cat serum (P11). 6 w.p.c. = 6 weeks post challenge.

Figure 7.2 Western blot analysis of sera from Leucogen vaccination experiment



This figure shows the western blot analyses of sera derived from the cats vaccinated with Leucogen (Virbac), which were taken at various stages during the course of the experiment. The blots were prepared from single well 10% or 15% gradient SDS-polyacrylamide gels. Samples containing native FeLV gp70 derived from the F422 cell line were electrophoresed for 90 minutes at 100 V. Electroblotting was then performed and the blots stored at -20 C. Strips (2 mm wide) were cut from the blots and were then probed with 1 in 20 dilutions of sera from the vaccinated and control cats. After 2 hours the strips were washed with PBS-tween and then incubated with biotinylated protein A, followed by a streptavidin-alkaline phophatase conjugate. The immunoblot strips were developed with NBT and BCIP in AP buffer. A control western blot was performed with a recovered cat serum (P11). 9 w.p.c. = 9 weeks post challenge. These results were generously provided by Dr. J. Christie and Mr. M. Golder (Feline Virus Unit).

al., 1988). Experimental cats were vaccinated with either the recombinant protein vaccine or a commercially available vaccine (Leucogen; Virbac) and then challenged by oronasal inoculation of FeLV-A. This route of inoculation was used as it mimics the natural route of infection (Rojko *et al.,* 1979b). In all three groups the challenge resulted in four out of the five cats becoming persistently viraemic. In the unvaccinated control group the remaining cat became transiently viraemic. In the group vaccinated with Leucogen the remaining cat became transiently p27 antigenaemic. In the group vaccinated with the experimental vaccine there was no evidence of FeLV replication in the remaining cat.

There are several precedents for using baculovirus derived recombinant proteins in vaccine studies. Recombinant glycoprotein D of herpes simplex virus (HSV) type 2 has been reported to elicit both humoral and cellular responses that protect mice against a subsequent lethal HSV challenge (Landolfi *et al.,* 1993). Similar responses were also observed against HSV type 1 proteins suggesting that cross-protection may be possible. However, not all recombinant proteins produced using baculoviruses are so effective. Vaccination against parainfluenza type 3 (PI-3) virus with either recombinant fusion (F) or haemagglutinin-neuraminidase (HN) proteins was only partially protective (van Wyke Coelingh *et al.,* 1987; Hall *et al.,* 1991). A chimeric FHN protein expressed in the baculovirus system induced higher levels of anti-PI-3 antibodies and better protection against experimental challenge compared with vaccination with either the F or HN proteins alone (Brideau *et al.,* 1993). Recombinant HTLV-1 *env*, when produced in insect cells, does not induce neutralising antibodies, though it does induce specific antibodies against synthetic peptides corresponding to important epitopes (Arp *et al.,* 1993).

A recombinant HIV *env* product, rgp160, has become the most intensively studied baculovirus-derived vaccine. This protein was extracted from the insect cells using sodium deoxycholate and then purified using gel filtration followed by lentil lectin affinity chromatography (Cochran *et al.* 1987; Dolin *et al.,* 1991). The vaccine preparation was adjuvanted with aluminium phosphate gel. This preparation was shown to be both safe and effective at inducing specific anti-gp120 antibodies in healthy humans (Dolin *et al.,* 1991). Neutralising antibodies were elicited in some subjects after several vaccinations. Antigen-specific lymphocyte proliferative responses were also elicited in humans and rhesus macaques vaccinated with this preparation (Keefer *et al.,* 1991; Newman *et al.,* 1992). Phase 1 clinical trials were also carried out with asymptomatic HIV positive individuals; the results were moderately encouraging (Redfield *et al.,* 1991). Antibodies appear to be directed at the carboxy terminus of the SU component rather than the putative neutralising V3 epitope (Newman *et al.,* 1992). This study also demonstrated the superior

adjuvanting properties of the purified Quil A derivative, QS-21. Incorporation of recombinant HIV gp120 into ISCOMS may improve the vaccine's immunogenicity further (Browning *et al.,* 1992). These results had suggested that the lentil lectin purified Bgp70-A protein when adjuvanted with Quil-A might serve as a vaccine against FeLV.

Leucogen, in contrast to the above vaccines, is an FeLV vaccine that contains a recombinant protein produced using a bacterial expression system (Marciani *et al.,* 1991). These authors reported the initial development of the vaccine from a truncated FeLV *env* gene which had been inserted into a temperature sensitive bacterial expression plasmid. The protein was initially solubilised with 6 M guanidine hydrochloride and then purified by gel filtration. The product was then redissolved in 8 M urea and a QS 21 adjuvanted preparation was used as an FeLV vaccine. Neutralising antibodies were induced in two out of seven cats prior to challenge and none of the vaccinated cats became either transiently or persistently viraemic. In contrast, three out of the four control cats became persistently viraemic, the remaining cat only became transiently viraemic. Clark *et al.* (1991) reported a more extensive trial which demonstrated that cats vaccinated with Leucogen only very occasionally produce virus neutralising antibodies. In this trial all the twenty control cats developed viraemia (six transiently) following challenge. Nine out of the twenty cats vaccinated with Leucogen did not develop viraemia; a further eight developed a transient viraemia. Others (Lehmann *et al.,* 1991) have shown that pre-existing FIV infection does not affect the response to this vaccination.

The results obtained from the vaccine trial reported in this chapter suggested that neither vaccine was effective at preventing FeLV infection. There was no evidence in any of the vaccinated cats of virus neutralising antibodies prior to challenge. There were high titres of anti-gp70 antibodies on western blots in the Leucogen vaccinated cats; however, four out of the five cats vaccinated with Leucogen became persistently viraemic and the remaining cat was initially antigenaemic. Antigenaemia, in the absence of viraemia, may indicate a low level of FeLV replication in this cat. It is therefore suggested that none of the cats vaccinated with Leucogen were fully protected against FeLV infection. The results of the western blotting suggested that the level of anti-gp70 antibodies determined by this technique is a poor indicator of the efficacy of the vaccine. The cats vaccinated with the baculovirus protein did not possess anti-gp70 antibodies prior to challenge and yet the same final result was achieved with this vaccine as with Leucogen.

No antigenaemia was demonstrated in the one cat that did not become viraemic in Group 1. It is therefore possible that this cat may have been protected by the

vaccination with Bgp70-A. However, as no markers of such protection were present prior to challenge this cat may have naturally recovered from the infection, the vaccine playing no part in the process.

The failure of both vaccines was unexpected. In particular, the failure of Leucogen was surprising considering previously reported vaccine trials (Marciani *et al.,* 1991; Lehmann *et al.,* 1991; Clark *et al.,* 1991). In the natural situation, even in high risk multiple cat households, only a proportion of cats become persistently viraemic. In experimental situations it is possible to induce persistent viraemia in every exposed cat using immunosuppression with corticosteroids or by infecting kittens at an early age (Hoover *et al.,* 1976; Rojko *et al.,* 1979a). It is therefore possible that the dose of challenge virus used in this experiment was too large and that the protection afforded by Leucogen was unable to eliminate the virus. This would suggest that the protection afforded cats by Leucogen in previously reported trials could be overcome by high viral doses. As it is not known what viral doses cats are exposed to in the natural situation it therefore follows that Leucogen may not provide any protection in certain situations. The number of cats becoming persistently viraemic following an experimental challenge can be used as a guide to the dose of virus used. A further trial should be conducted with a larger number of cats and a dose of virus such that 60%-80% of the controls become persistently viraemic.

The failure of the cats vaccinated with the recombinant glycoprotein may also be due to an excessive challenge. It is also possible that insufficient antigen was present. Possible improvements in the production of the protein have been discussed in Chapter 4. A third explanation could be a failure of the adjuvant. Quil A contains a mixture of glycosides extracted from saponin which is isolated from bark. As such, a certain heterogeneity in composition is to be expected. Other adjuvants which could be considered in future trials include muramyl dipeptide derivatives (Byars and Allison, 1988) and ISCOMs (Morein *et al.,* 1984; Osterhaus *et al.,* 1985; Osterhaus *et al.,* 1989)

The second adjuvant that was included in the vaccine was aluminium hydroxide. Aluminium salts have been widely used in both medical and veterinary vaccines (Gupta *et al.,* 1993). They are thought to be mild adjuvants which may therefore be inadequate for recombinant soluble protein vaccines. Recently it has been demonstrated that a failure of proteins to adhere to the aluminium salts may be the cause of this problem (Skea and Barber, 1993). To correct this deficit, antibody mediated adhesion of the proteins to the aluminium was attempted with encouraging results. It would be sensible to measure the decrease in detectable protein following preparation of any aluminium salt adjuvanted vaccine.

In conclusion, this vaccine trial demonstrated that the protection afforded to cats by vaccination with Leucogen is, at best, only partial. Leucogen did induce antibodies that detected gp70 in western blotting experiments. Such antibodies did not confer protection against the development of viraemia. In contrast the baculovirus derived recombinant protein preparation did not induce anti-gp70 antibodies that were detectable on western blots. However, this vaccine may have prevented the development of viraemia in one cat, though the cat may have recovered naturally. Further vaccine studies should be performed with this protein. Investigations into the role of cytotoxic T cells in the protection of vaccinated cats are of particular importance. Despite the many reports of FeLV vaccines that have been produced over the years, there is still a need for an effective, commercially viable, FeLV vaccine.

Chapter 8. General Discussion

This chapter contains a general discussion on the nature and significance of the studies presented in this thesis. Suggestions for future work are made in the relevant parts of the chapter. A selection of recent innovations that I consider may be of value in the study of FeLV pathogenesis and vaccination are also presented.

8.1 The Production of the Recombinant Surface Glycoproteins

This thesis describes the production, purification and analysis of recombinant FeLV surface glycoproteins. The proteins were produced in the baculovirus expression system from truncated *env* genes of FeLV-A and FeLV-C/Sarma and two chimeric FeLV-A/C chimeras produced by Rigby (1989). The recombinant proteins were termed Bgp70-A, Bgp70-C, Bgp70-VC and Bgp70-215, respectively. Truncated *env* genes were used so that the resulting protein would be secreted. It is known that retrovirus surface glycoproteins are only secreted in quantity if the transmembrane portion of the TM protein is deleted and the amino terminus of TM is left intact (Einfeld and Hunter, 1988). The identity of the putative transmembrane portion of the FeLV TM protein is known (Stewart *et al.,* 1986), but the minimal requirement for the amino terminus sequences is not. During the course of the project the production of an FeLV surface glycoprotein product in the baculovirus system was reported (Thomsen *et al.,* 1992). These authors expressed a full length *env* gene in Sf9 cells and found that the protein was retained within the cells.

The recombinant proteins were efficiently exported and could be detected in the culture fluid of infected cells. This result was consistent with those of similar experiments using HIV *env* (Wells and Compans, 1990). The production of secreted surface glycoprotein was useful as it simplified subsequent purification procedures. Thus disruption of infected insect cells by detergent to obtain recombinant protein (Rusche *et al.,* 1987), which may affect the functional integrity of that protein, was avoided. Prior to purification, the supernatants were concentrated by ultrafiltration. An attempt to purify the proteins using a nickel nitrilotriacetic acid-agarose adsorbent (Hochuli *et al.,* 1987) was unsuccessful. Immunoaffinity purification was not attempted due to the lack of a suitable quantities of the necessary antibody reagents. However, lentil lectin affinity chromatography was found to be an effective method of purifying the recombinant proteins. The only major contaminant in the preparations purified in this way was believed to be the major baculoviral envelope protein, gp64 (Volkman, 1986). An anti-gp64 monoclonal antibody could be used in western blot assays to examine this hypothesis; several such antibodies are available (Roberts and Manning, 1993).

Having established a protocol for the production of the proteins, several large batches of supernatants were concentrated and purified using lentil lectin affinity chromatography. These stocks were used for a number of experiments which examined the biological properties of the proteins. The recombinant proteins were found to be approximately 80 kDa in size and they were not cleaved. The quantity of protein produced was initially believed to be low in comparison to reported values for other retrovirus surface glycoproteins expressed in this system (Rusche *et al.,* 1987; Hu *et al.,* 1987; Wells and Compans, 1990; Thomsen *et al.,* 1992; Arp *et al.,* 1993). However, it was suggested in Chapter 4 that the difference may be apparent rather than real. As the recombinant protein preparations were contaminated with the major baculoviral envelope protein, the exact quantity of recombinant protein was never determined. Further truncation of the amino terminus of the TM gene could be performed to define the minimal requirement for SU secretion. This could assist the production of SU as the shorter transcripts may allow the production of larger quantities of protein.

Recombinant proteins expressed in the baculovirus system are often incompletely glycosylated (King and Possee, 1992) and FeLV SU is no exception. Deglycosylation of the recombinant protein with PNGaseF demonstrated that the proteins were glycosylated, but not to the same extent as their native counterparts. The difference between the predicted deglycosylated size of the recombinant protein and the value observed by deglycosylation with PNGaseF was thought to be due to the presence of small quantities of O-linked carbohydrate entities. As reported by others, this difference was also observed in the native protein (Pinter and Honnen, 1988). Formal proof would require the use of a neuramidase prior to deglycosylation.

The lentil lectin affinity purified preparations were analysed by western blotting and ELISAs. The results of these experiments indicated that the recombinant proteins were immunologically similar to their native counterparts. It was also demonstrated that subgroup-specific monoclonal and polyclonal antibodies could be used to differentiate between Bgp70-C and Bgp70-A in ELISAs. The two chimeric proteins, Bgp70-VC and Bgp70-215, were indistinguishable from Bgp70-A. These results suggested that the proteins could be used to investigate the nature of some aspects of the initial virus / host interaction.

8.2 Towards the Identification of a Neutralising Epitope for FeLV

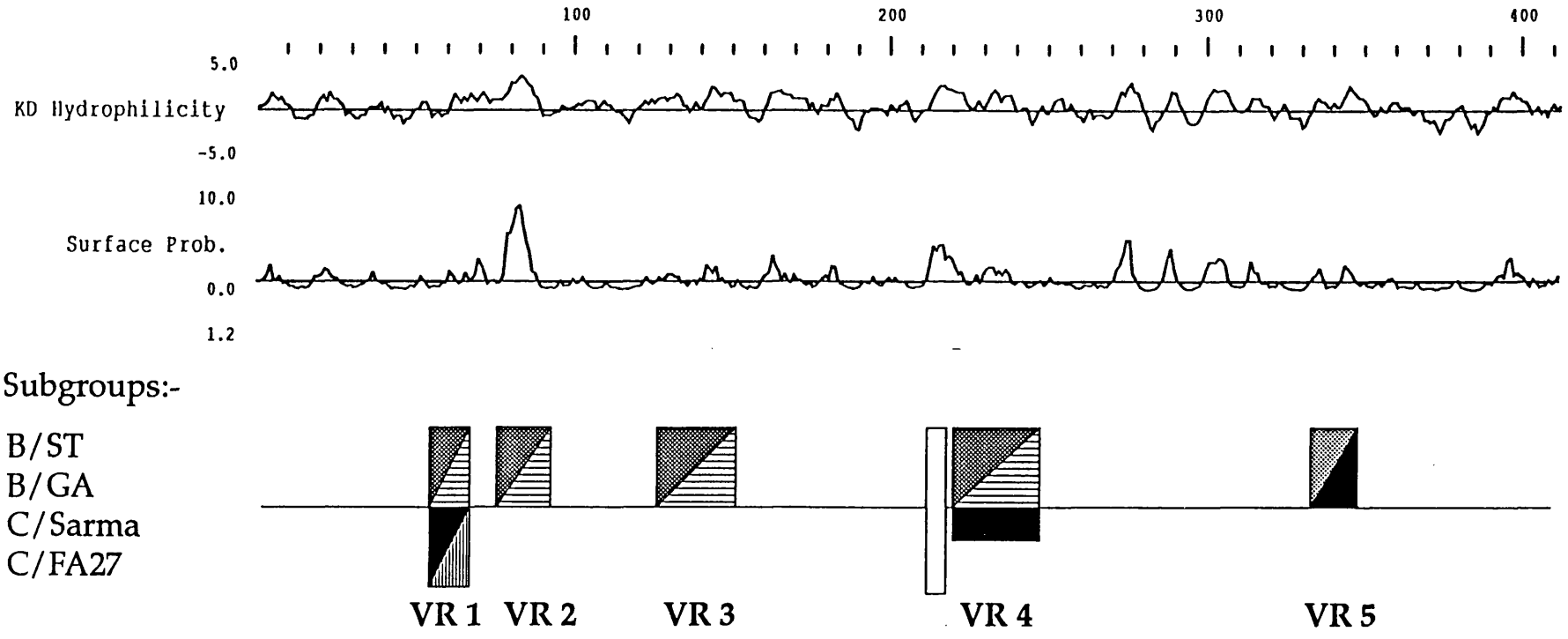
It has been demonstrated that the neutralising antibodies produced by cats are, at least partially, subgroup-specific (Russell and Jarrett, 1978b). If the FeLV-A/C

phenotype is mostly determined by VR 1 (Rigby *et al.,* 1992) then it is logical to suggest that VR 1 may be involved in the binding of neutralising antibodies. The neutralisation inhibition assays described in Chapter 5 demonstrated that the recombinant protein Bgp70-A was able to block the action of neutralising antibodies. Furthermore, it was shown that Bgp70-C was unable to block the action of neutralising antibodies found in sera derived from cats that had been naturally infected with FeLV-A. However, the two FeLV-A/C chimeric proteins, Bgp70-VC and Bgp70-215, were also able to block the neutralising activities of these sera. This provided the conformation that the subgroup determining and neutralising antibody binding sites of FeLV are distinct (Russell and Jarrett, 1978b). The subgroup-determining VR 1 region appears to play little or no part in the binding of neutralising antibodies.

The nature of neutralising epitopes is known only for some viruses. Examples include the V3 loop of HIV (Skinner *et al.,* 1988; Javaherian *et al.,* 1989) and the loop of influenza virus haemagglutinin (Wiley *et al.,* 1981). In so far as generalisations can be made from these examples, it would appear that neutralising epitopes are composed of contiguous amino acids, albeit often arranged in a particular conformation. Other regions of the molecule may play a peripheral role in binding. It may be possible therefore to identify a region or regions that are altered in Bgp70-C which may serve as binding sites for neutralising antibodies.

Figure 8.1 correlates data derived from two sources. The surface probability and hydrophilicity plots of FeLV-A SU were derived from the output of the PLOTSTRUCTURE program which is part of the GCG package for the VAX network (Devereux *et al.,* 1984; Jameson and Wolf, 1988). These plots give an indication of the properties of a given segment of a protein based in the primary amino acid sequence. The hydrophilicity plot indicates the degree of expected hydrophilicity in a 6 to 10 amino acid window and can therefore be used as an indication that the particular sequence is located on the surface of the protein. Highly charged clusters of amino acids are frequently found to be exposed to the aqueous environment, whereas strongly hydrophobic clusters are often incarcerated in the centre of the protein (Jameson and Wolf, 1988). The surface probability plot is a similar measure derived from the side chain solvent accessibility values of the individual amino acids. Under the two plots is a line corresponding to the FeLV-A sequence. Above and below the lines are boxes which represent variable regions (VRs), as defined by Rigby (1989). Horizontal lines separate sequences that are dissimilar, diagonal lines separate sequences that are the same. When sequences are homologous to A they are not represented. Therefore, in variable region 5 (VR 5) FeLV-C/Sarma and FeLV-B/Snyder-Theilen differ from FeLV-A, but FeLV-

Figure 8.1 PLOTSTRUCTURE diagram of FeLV SU with proposed variable regions



This diagram shows the amino acid residue number, Kyle-Doolittle hydrophilicity and surface probability plots of FeLV-A/Glasgow-1 SU generated by the PLOTSTRUCTURE program of the GCG package (Jameson and Wolf, 1988; Devereux *et al.*, 1984). Under these plots is a line corresponding to the FeLV-A/Glasgow-1 sequence. Above and below the line, the variable regions (VR) of FeLV SU (as defined by Rigby, 1989) are shown as shaded areas. The areas separated by horizontal lines differ, but those separated by angled lines do not. In VR5 therefore, FeLV-C/Sarma and FeLV-B/Snyder-Theilen (ST) are identical and differ from FeLV-A/Glasgow-1, but FeLV-B/Gardner-Arnstein (GA) and FeLV-C/FA27 are similar to FeLV-A/Glasgow-1. The unshaded box represents the neutralising epitope that is recognised by the monoclonal antibodies C11D8 (Grant *et al.*, 1983; Elder *et al.*, 1987) and 3-17(Weijer *et al.*,1986; N.Spibey, personal communication). This epitope is common to all FeLV sequences used in this diagram.

B/Garner Arnstein is the same as FeLV-A.

Predictions of higher orders of structure based on the primary peptide sequence are frequently misleading. However, the diagram does correctly identify the neutralising epitope recognised by the monoclonal antibody C11D8 (Grant *et al.*, 1983; Nunberg *et al.*, 1984a; Elder *et al.*, 1987) as having a high surface probability. The monoclonal antibody 3-17 (Weijer *et al.*, 1986) described throughout this thesis also binds to this region, though it does not interfere with the binding of C11D8 (N.Spibey, personal communication). This prediction is to be expected as several such antibodies have been produced (Weijer *et al.*, 1986; Elder *et al.*, 1987). These antibodies neutralise the virus *in vitro* in a subgroup independent fashion. As cats produce subgroup-dependent neutralising antibodies, it is suggested that this epitope is not recognised in the cat.

If VR 1 is not directly involved in the binding of neutralising antibodies, and yet the neutralising antibody binding site(s) do vary between isolates, then the diagram presented in Figure 8.1 may help to decide which other regions of FeLV SU are likely to be involved in the binding of neutralising antibodies. It is suggested that the neutralisation epitopes are likely to be those regions of the SU protein which differ between FeLV-A and FeLV-C/Sarma. It is possible, however, that changes in the tertiary protein structure of other regions of SU may be caused by mutations in FeLV-C. Notwithstanding this caveat, by comparison of the sequence data and predicted surface probabilities, it is possible to suggest that the most likely sites for neutralising epitopes are VR 4 and VR 5. They are both heterologous between FeLV-A and FeLV-C/Sarma. They also are hydrophilic and possess moderately high surface probabilities.

FeLV-B/Gardner-Arnstein (FeLV-B/GA) is homologous to FeLV-A in VR 5; but, the ability of FeLV-A sera to recognise FeLV-B/GA appears to be unknown. Therefore, it is not possible to discriminate further which region may be involved. The production of FeLV-B/GA SU in the baculovirus expression system and subsequent use in the neutralisation inhibition assay could help to solve this problem. However, it would be simpler to obtain FeLV-B/GA and perform a neutralisation assay. As FeLV-B/GA differs in several other regions from FeLV-A, formal proof will require the generation of VR4 and VR5 chimeras.

Using site directed mutagenesis to alter the nucleotide sequence of the *env* gene, convenient restriction enzyme sites could be introduced upstream of VR 4 and also between VR 4 and VR5. This would allow the generation of VR 4 or VR 5 chimeric recombinant proteins. VR 4 plus VR 5 chimeric proteins would also be created in the process. Such proteins could then be used in neutralisation inhibition

assays to define further the regions that are involved in neutralising antibody binding. Specifically, by mutation of nucleotide residues 811 and 812 (cytidine to thymidine and cytidine to adenosine) (Stewart *et al.,* 1986) an *Xba* 1 site can be introduced. Similarly, site directed mutagenesis of nucleotide residue 1019 (cytidine to guanosine) introduces an *Xho* 1 site, without affecting the resulting amino acid sequence. These restriction enzyme sites could also be introduced into FeLV-C/Sarma using the same oligonucleotide primers. The introduction of the *Xba* 1 site into FeLV-B/GA could also be performed with this primer, but a slightly different oligonucleotide primer would preserve the guanine residue at nucleotide residue 813.

Although it would appear the either or both of VR4 and VR5 are part of the neutralising epitope the involvement of other, constant, regions cannot be discounted from these experiments and observations.

8.3 FeLV Cell Surface Receptors

The ability of the recombinant proteins to block FeLV infection in a subgroup specific manner was employed in an attempt to define cellular receptors for FeLV. The attempt failed. However, the experiments demonstrated that co-immunoprecipitation may not be the most appropriate way to identify as specific cell surface receptor. Recent experiments defining the receptors for two retroviruses used recombinant retroviruses (Albritton *et al.,* 1989; O'Hara *et al.,* 1990). This may be a more sophisticated and reliable method for performing such studies, though the time and financial resource commitments should not be underestimated.

On the basis of superinfection interference studies with retroviral pseudotypes, it has been suggested that groups of retroviruses share common receptors on human cells (Sommerfelt and Weiss, 1989). Gibbon ape leukaemia virus (GALV) and FeLV-B are members of one such group. With the discovery of the gibbon ape leukaemia virus receptor (GLVR1) it was possible to confirm that FeLV-B does indeed use the same phosphate transporter protein (Takeuchi *et al.,* 1993). However, the fine binding specificities of the two viruses differ. Mutation of three amino acids within a critical nine amino acid region of GLVR1 induces resistance to FeLV-B but not GALV (Tailor *et al.,* 1993). A similar situation has been demonstrated in the MuLV system (Eiden *et al.,* 1993). These authors reported that subtle differences in the ecotropic murine leukaemia virus (E-MuLV) receptor protein could effectively block the entry of the Moloney strain of E-MuLV into a murine fibroblast cell line. In contrast, two other E-MuLVs, Friend MuLV and Rauscher MuLV, were able to infect the cells. It seems likely that such subtle differences account for the ability of FeLV-C, but not FeLV-A, to infect guinea pig

cells. It has not yet been demonstrated that FeLV-B uses the same receptor on feline cells as on human cells. This study should be performed to assess the significance of the findings made with human cells. It would be useful to generate a recombinant FeLV-B SU in the baculovirus system and to define a method by which it could be used to identify GLVR1 on human cells. Success would greatly encourage further cell surface receptor studies using the proteins detailed in this thesis. Unfortunately, no other retroviruses appear to use the same receptor as FeLV-C on human cells (Sommerfelt and Weiss, 1989) and FeLV-A does not infect non-feline cells, so it is unlikely that the receptors for either subgroup will be identified in the same, co-incidental, manner as the FeLV-B receptor.

The specificities of retroviruses for their receptors may explain the existence of endogenous retroviruses. It is possible that xenotropic retroviruses, such as RD 114, arose from ecotropic viruses which infected the germ line of a host and subsequently suffered from a mutation in the relevant VCSR gene. This would imprison the virus, albeit in the host germ line, unable to spread horizontally, except in experimental studies to certain heterologous species.

With the identification of several VCSRs as common, important host cell proteins the current interest in VCSRs will continue. However, the original interest in VCSRs as targets for anti-viral therapy seems to become less relevant with each new VCSR that is identified. The biology of the virus should be taken into account before strenuous efforts are made to identify its VCSR. FeLV-A replicates in many of the tissues of its natural host. It is neutralised by naturally produced antibodies. It is suggested that, in attempting to control FeLV infection, the importance of identifying the VCSR is less than that of generating an effective vaccine.

8.4 FeLV Vaccination

The results presented in this thesis illustrate some of the difficulties involved in FeLV vaccine trials and demonstrate that a commercially available FeLV vaccine is of questionable efficacy. The failure of the experimental vaccine should not discourage the further investigation of Bgp70-A as an immunogen. Greater quantities of protein or more frequent administration should be used in future trials.

The aim of FeLV vaccination should be to prevent FeLV infection entirely. A cat that has recovered from FeLV infection usually possesses virus neutralising antibodies, which protect it against subsequent challenge (Jarrett *et al.,* 1977; Hoover *et al.,* 1977; deNoronha *et al.,* 1978; Salerno *et al.,* 1978). There are no other characterised markers for the immune cat. Until there are, the induction of prechallenge virus neutralising antibodies must be regarded as the ideal that FeLV

vaccine researchers should strive to attain. Since the first report of an FeLV vaccine trial, many attempts have been made to demonstrate an effective FeLV vaccine. Only one experimental vaccine has been shown to consistently induce virus neutralising antibodies against FeLV (Osterhaus *et al.,* 1985; Osterhaus *et al.,* 1989).

Unfortunately, there has been no consensus between these trials as to the challenge protocols used, the presentation of results, interpretation of data or the inferences drawn. Some investigators, in an attempt to overcome the age-related susceptibility of cats to FeLV, have challenged cats by intraperitoneal injection (Marciani *et al.,* 1991; Clark *et al.,* 1991). Others have supplemented challenge with immunosuppressive therapy (Sebring *et al.,* 1991). FeLV is not thought to normally enter the cat by direct inoculation (Rojko *et al.,* 1979b) and the use of intraperitoneal inoculations may by-pass important mucosal immune systems. It is suggested that the use of intraperitoneal inoculation as a challenge protocol is less relevant than oronasal administration of the virus, without corticosteriod treatment to 12-16 week old SPF cats (Osterhaus *et al.,* 1985; Tartaglia *et al.,* 1993). If kittens of this age are used, the practice of administering immunosuppressive therapy post-challenge (Lewis *et al.,* 1981; Mastro *et al.,* 1986; Haffer *et al.,* 1987; Tizard and Bass, 1991; Hines *et al.,* 1991) is unnecessary. In any case, such cats represent the major at-risk group and so vaccination studies should be focussed on them. In the natural situation young cats will probably be exposed several times to low doses of FeLV. A challenge system which effectively mimics this feature would be an improvement on conventional challenges. However, the sole experiment performed with repeated low dose challenge over a short period of time (Pedersen *et al.,* 1986) only induced persistent viraemia in six out of seventeen controls.

An in-contact challenge system using persistently viraemic cats has been used by several groups with varying success (Pedersen *et al.,* 1979). Such trials require the use of many cats and the effects on the controls are less predictable than in other challenge systems. The use of naturally infected cats from a rural environment in conjunction with SPF cats (Legendre *et al.,* 1990) would be unacceptable in the UK. The subsequent development of respiratory diseases as well as deaths from feline infectious peritonitis could have been avoided by using SPF cats that had been experimentally infected with FeLV. The use of uninfected outbred cats as indicators in multicat households containing FeLV excreting cats has been reported by others (Pollock and Scarlett, 1990; York and York, 1991). The results of these trials are valid only if the indicator cats have no evidence of previous infection with FeLV. The presence of anti-FOCMA antibodies is thought to be a good indicator of such exposure (Jarrett, 1984; Pollock and Scarlett, 1990). It is suggested that

assaying the indicator cats only for virus neutralising antibodies is insufficient (York and York, 1991).

There are also inconsistencies in the interpretation of the results of vaccination experiments. Some investigators regard transiently infected cats as being protected (Pedersen *et al.,* 1986). However, if a vaccinated cat develops a transient viraemia following experimental infection then the vaccine has not prevented viral replication. At a recent FeLV vaccination conference, the report of the panel was in favour of regarding experimentally vaccinated cats which become transiently viraemic following challenge as being vaccine failures (Anonymous, 1991).

The small number of cats in most trials has precluded the use of meaningful measures of statistical significance. The concept of the 'preventable fraction' (PF) has become popular in recent years (Pollock and Haffer, 1991; Sebring *et al.,* 1991; Clark *et al.,* 1991). This value is obtained by expressing the difference between the number of vaccinates and controls that become viraemic as a fraction of the number of viraemic controls. This means that if a low number of the controls become viraemic then the significance of any drop amongst the vaccinates is greatly magnified. In fact, the reverse is more likely to be the case. It is suggested that analyses using the PF should only be applied when more than 80% of the controls become viraemic.

The task of vaccine researchers should be to express the neutralisation epitope of FeLV SU in its native conformation and present it to the immune system of the cat in an immunogenic form. Identification of the epitope may assist this process. The work detailed in this thesis suggests a method by which this could be accomplished. Though identification of the neutralisation epitopes of a virus is not a prerequisite for successful vaccine manufacture, when traditional approaches have failed, or are considered too risky, such knowledge is invaluable in the rational design of subunit vaccines. In the future, research into the T cell mediated responses to FeLV may demonstrate other regions of FeLV SU which are of importance in preventing the development of persistent viraemia. Such regions will also be included in subunit FeLV vaccines of the future.

Subunit vaccines may not represent the optimum method of vaccinating against FeLV. Recently, a live SIV vaccine, attenuated by a deletion in the *nef* gene, has been shown to be effective at preventing infection (Daniel *et al.,* 1992). A disadvantage of this approach is the potential for the attenuated virus to recombine with endogenous retroviral elements to produce a fully infectious, pathogenic variant. Retroviral recombination is known to be both frequent and ordered (Hu and Temin, 1990). Further investigation into the mechanism of retroviral recombination

may reveal methods by which the process can be prevented. Another problem is the potential for transduction of cellular oncogenes by the attenuated virus, which must integrate into the host genome to be able to replicate. Transduction of cellular oncogenes is thought to be due to the formation of read-through transcripts resulting from inefficient viral polyadenylation signals (Swain and Coffin, 1992). Definition and alteration of these signals may reduce the incidence of such transcripts. This type of approach to vaccination, despite these potential problems, should not necessarily be discounted. The use of an attenuated virus as an inactivated virus vaccine could reduce the harm caused by, though not the risk of, inadequate inactivation. This approach can only be effective in virus systems, such as FIV and SIV, that have non-essential, potentiating genes. No such genes are known for FeLV. However, by creating a VR 1 mutant that could not replicate in cat cells, but could replicate in another species cells, such an attenuated vaccine could be generated. The attenuated virus could also be used for the production of an inactivated FeLV vaccine. Inactivated FeLV vaccines are known to be efficacious (Hoover *et al.,* 1991; Tizard and Bass, 1991), however they must be shown to be completely inactivated (Shibley *et al.,* 1991). An inactivated, attenuated virus would introduce an extra element of safety into such preparations.

Another recent development is the use of plasmid DNA to induce protective immunity (Tang *et al.,* 1992; Robinson *et al.,* 1993). The rationale underlying this innovation states that DNA introduced into host cells by whatever means can, potentially, stimulate immunity. Plasmid DNA cannot produce infectious progeny and so is inherently safer than live virus vectors. If sufficient cells could be transfected *in vivo* with such a plasmid containing the foreign gene then the need for viruses as vectors is obviated. An additional advantage is the induction of heterologous protection due to cytotoxic T cell activation (Ulmer *et al.,* 1993). This technique may yield useful results in the FeLV system. However, a number of problems will need to be solved before an effective, commercially viable, DNA vaccine against FeLV is available. For example, developing an effective carrier, that is easily administered in practice, for the DNA will require much careful thought. Recombinant viruses, when used as vaccine vectors, are effective means of introducing relevant DNA into cells. Such vectors are capable of replication and recombination in the host and so there are concerns regarding their safety. The use of non-replicating viral vectors represents an improvement in the development of such vaccines (Tartaglia *et al.,* 1993).

This thesis also reports the production of recombinant FIV SU in the baculovirus system. Recently, the expression of the entire FIV *env* gene and subsequent use of the product for immunodiagnosis has been reported (Verschoor *et al.,* 1993). The

use of the recombinant surface glycoprotein of FIV, Bgp100, as a FIV vaccine deserves further study. A better yield of recombinant protein may be obtained by including the sequences coding for the amino terminus of the FIV TM protein in the transfer vector. Progress towards the identification of a linear neutralising epitope within FIV SU has recently been reported (Lombardi *et al.*, 1993).

8.5 Conclusion

It is suggested that the principal use of Bgp70's will be in the analysis of the immune response to FeLV infection. Future studies involving manipulation of the envelope gene and subsequent expression in the baculovirus system should be able to rapidly produce recombinant chimeric proteins, free of infectious virus, for the localisation of the major neutralising epitopes of FeLV. Also, the products of envelope genes which do not produce helper-independent progeny could be examined in isolation from their helper virus.

A secondary use of the Bgp70s may be to identify, albeit probably indirectly, FeLV cell surface receptors. The production of anti-receptor antibodies may be facilitated by using cell membrane proteins, purified by Bgp70 affinity chromatography, as immunogens. The recombinant proteins might also be used to screen cDNA libraries containing cat genomic DNA.

This thesis demonstrates the potential for the further analysis of several aspects of retrovirus pathogenesis using recombinant surface glycoproteins. The effect of manipulations of the *env* gene can be rapidly assessed using this system. In particular, site directed mutagenesis of other regions of the *env* gene could be undertaken in an attempt to further define the neutralising epitopes of FeLV. The identification of these epitopes will greatly assist the design of an truly effective FeLV vaccine.

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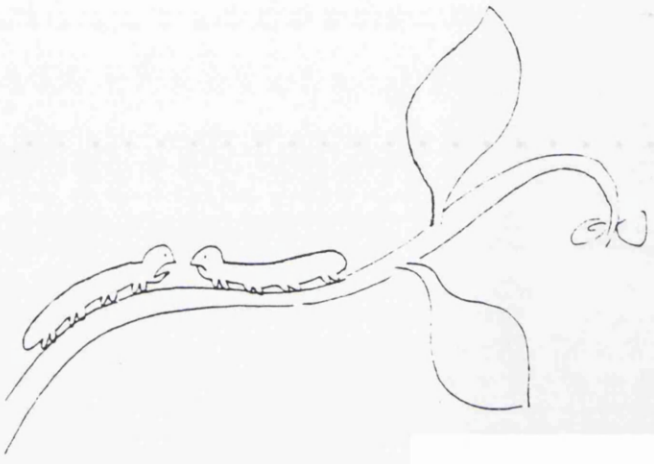
Endpiece



"You know what worries me? I've never seen an old caterpillar."