THE POSITIONAL CLONING OF Fv-1
A MOUSE RETROVIRAL RESTRICTION GENE

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Abstract

The mouse \textit{Fv-1} gene restricts the replication of murine leukaemia viruses (MuLVs) and has been mapped to the distal region of chromosome four. This gene somehow prevents integration of viral DNA into the host genome.

Several genes, notably four endogenous proviral elements (Xmvs) and \textit{Pnd} were linked to \textit{Fv-1} and so offered a route to clone the gene by a positional approach. A backcross was analysed to position \textit{Fv-1} more accurately relative to the linked genes. This data revealed that \textit{Pnd} and \textit{Xmv-9} were within \~1,200 kilobases of \textit{Fv-1}. Yeast artificial chromosome (YAC) clones carrying linked genes were isolated. Attempts were made to generate a contig of the area, this but was complicated by the presence of mouse B1, LINE and zinc finger repeats, and an area of instability in the clones.

YACs judged to lie closest to \textit{Fv-1} were introduced, by fusion, into mouse cells. It was anticipated that the presence of \textit{Fv-1} on a YAC would be detected by the ability to restrict an infectious virus. Of the cell lines tested, L cells gave the best fusion results, with a high frequency of complete YAC integration. The L cells produced their own reverse transcriptase (RT) from an endogenous virus. The virus infectivity assay, which monitored virus production by RT activity, was unusable. An alternative assay was developed which used colony formation as a measure of infection. This led to the identification of a 190 kilobase YAC which conferred \textit{Fv-1} activity.

Identification of the \textit{Fv-1} gene is currently underway. Cloning \textit{Fv-1} will allow the biochemical mechanisms involved in virus restriction to be investigated, and will offer a wider insight into the mechanisms of retroviral integration. The implications for the prevention of human retroviral disease are potentially important.
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Chapter 1
Introduction

The mouse $Fv-1$ gene restricts the replication of murine leukaemia viruses (MuLVs) and has been mapped to the distal region of chromosome four. The aim of this project was to clone the gene which would enable the molecular events causing restriction to be investigated.

The introduction will deal firstly with the nature of the MuLVs and their life cycle. This will be followed by an account of the discovery of these retroviruses and their ability to induce tumour formation and leukaemogenesis in the mouse. Mouse genes play a major role in determining susceptibility to infection by these viruses and the existence of several such genes has been demonstrated. Of particular importance is the mouse retroviral restriction gene $Fv-1$, whose presence has been recognised for over twenty years. The mechanisms by which these genes interfere with viral replication has been explained in some cases, but the way in which $Fv-1$ functions remains unknown. An account of the discovery of $Fv-1$ and the other viral restriction genes will be presented. Next, the effect that $Fv-1$ has upon the outcome of infection and the possible mechanisms by which this is achieved will be discussed. Finally, the proposed means to clone and study $Fv-1$ will be explained.

1.1 Overview of Retrovirus Replication

In order to understand the mechanism by which the $Fv-1$ gene product might interact with a leukaemia virus, it would be useful to outline the genomic structure of the retrovirus and the basic events which take place during the retrovirus life cycle. The particular aspects discussed here concerning the viral
genome apply specifically to Murine Leukaemia Viruses, but the general principles apply equally well to other retroviruses. The information in sections 1.1.1 to 1.1.3 has been drawn together from extensive reviews on retrovirus biology (Coffin, 1990; Varmus, 1988; Varmus and Brown, 1989).

The MuLVs are a group of type C retroviruses in the sub-family Oncovirinae. They induce a number of different types of leukaemias and lymphomas in mice. However, no extracellular, or exogenous, examples of the C-type viruses have been isolated from humans.

### 1.1.1 The Structure of the Virus

The extracellular virus particles are ~90-100 nm in diameter and consist of an outer envelope with an inner matrix coat below, surrounding a nucleoprotein core consisting of an icosahedral shell containing genomic RNA, protein and other nucleic acids. The genomic RNA consists of two identical single strands 7-9 kb in length. These are positive-sense molecules (like cellular mRNAs) and have other features characteristic of eukaryotic mRNAs. They include a 5' capping group, methylation of some adenine residues and a stretch of about 200 A-residues at the 3' end. Associated with the RNA are single small tRNAs which initiate reverse transcription.

### 1.1.2 The Life Cycle

The basic retrovirus life cycle can be seen in Figure 1.1. Briefly, the extracellular virus particle enters a susceptible host cell via the attachment of viral envelope proteins to a host-encoded transmembrane receptor. The particle becomes internalized by receptor-mediated endocytosis. It has been demonstrated that entry of MuLV is pH independent (McClure et al, 1990).
The next step is reverse transcription, a complex process which generates a double stranded DNA molecule from the RNA template. The DNA is transported in to the cell nucleus where it integrates stably into the host genome and is then known as a provirus. Reverse transcription and integration into the cellular genome occur with apparently little or no help from the host cell. Both processes occur within a nucleoprotein complex left over from the infecting virion.

During reverse transcription, sequences at the 3' and 5' ends of the viral RNA are duplicated to generate identical long terminal repeats (LTRs) at both ends of the double stranded viral DNA. The LTRs contain cis-acting sequences necessary for integration and expression of the virus. The integrated provirus is dependent upon the host for transcription and translation in order for the viral life cycle to proceed. The final stages of the life cycle are the assembly and budding of a new virus particle. The nature of
these processes are poorly understood; however, capsid assembly, incorporation of full-length RNA genomes and budding appear to occur simultaneously.

1.1.3 The MuLV Genome and Its Expression After Integration

The genomic organisation of the provirus can be seen in Figure 1.2. The ends of the viral genomic RNA consist of a short, ~60 bp repeats (R) which are required for DNA strand transfer during reverse transcription. The U5 region (~75 bp) at the 5' ends and the U3 region (~500 bp) at the 3' end, contain signals for synthesis and polyadenylation of viral RNA, reverse transcription and signal recognition by the integration machinery. These are duplicated during reverse transcription to form the LTRs. A primer binding site of 18 bp follows U5 and binds the minus-strand tRNA primer to allow initiation of reverse transcription. A leader of ~475 bp follows which contains a splice donor site for mRNA processing. The packaging signal which specifies incorporation of the genomic RNA into budding virions is also located in this area, but probably extends 3' into the coding region.

![Figure 1.2 Genomic Organization of MuLV](image)

Figure 1.2 Genomic Organization of MuLV
The coding regions of MuLV are known as \textit{gag}, \textit{pro}, \textit{pol} and \textit{env}. The provirus is transcribed using cellular RNA polymerase II into a single RNA precursor which serves as both genomic RNA for a new virus particle and as mRNA for the \textit{gag}, \textit{pro} and \textit{pol}. Some transcripts are spliced to give an mRNA which codes only for \textit{env}. The way in which the frequency of splicing is controlled is not understood. All of the mRNAs are transported to the cytoplasm where translation occurs. The spliced mRNA encoding the \textit{env} (envelope) gene codes for a large 70 kDa surface glycoprotein (gp70 or SU) and a smaller non-glycosylated transmembrane protein of 15 kDa (TM or p15). gp70 is the cell receptor-binding molecule which initiates receptor-mediated endocytosis required for uptake of the virus into the cell. It is this molecule to which neutralizing antibodies can be elicited and defines the host range (see below). The smaller hydrophobic TM protein is associated with gp70 and is probably involved in mediating fusion of the virus and the host cell membrane.

Translation of the full length mRNA is more complicated. Ribosomes bind upstream of \textit{gag} in the leader sequence but then move to the \textit{gag} initiation codon where translation begins. At the end of \textit{gag}, the ribosome encounters an amber translational terminator and 95% of translation stops. The \textit{gag} polyprotein is then cleaved to form four protein products. The first is the matrix protein (MA), also known as p15 because it has a molecular weight of 15 kDa. Following this is an acidic phosphoprotein (p12) of unknown function. Next is the major capsid polypeptide (CA or p30), then a small basic nucleoprotein (NC or p10) which binds virion RNA and is implicated in RNA packaging and RNA dimer formation.

During translation, at a frequency of about 1 in 20, the \textit{gag} amber stop codon is suppressed and read-through into \textit{pro} and \textit{pol} occurs, both of which are in
the same reading frame as gag. This mechanism also occurs at the pro-pol boundary. The pro gene encodes PR, a protein which cleaves gag and pol precursor proteins. The pol gene encodes reverse transcriptase (RT), a single polypeptide of 70-80 kDa of which there are probably several per virion particle. It also encodes integrase (IN or p46). The end of the pol reading frame overlaps with env. The translation termination signals ensure that the various proteins are synthesised in the correct proportion relative to one another and the bypassing of which facilitates the combined incorporation of the pro and pol encoded-enzymes into the mature virion particle.

1.1.4 Endogenous Retroviruses
A cell is not killed following infection by a MuLV. Having integrated into the host genome, the provirus directs production of progeny virus. If a stem cell is infected, each daughter cell will receive a copy of the provirus. If the germ line becomes infected, then offspring will inherit the provirus in a stable Mendelian fashion (Coffin, 1982; Stoye and Coffin, 1985). Such integrated viruses are termed endogenous retroviruses and may be found throughout the chromosomes of probably all vertebrate species.

Endogenous retroviruses are usually transcriptionally silent and are often found to be defective due to deletions and point mutations acquired over time during the course of breeding of the host. Many are likely to have mutated prior to integration. Those endogenous retroviruses which are expressed and replication competent are not generally thought to be pathogenic in the host, however exceptions do exist (see section 1.3.1). If pathogenic effects were common and severe, the endogenous viruses responsible would not be maintained in the host species for any length of time (Coffin, 1990). The insertion and maintenance of endogenous
retroviruses can also cause mutations, for example the dilute (Jenkins et al, 1981) and hairless mutations (Stoye et al, 1988) in mice.

1.1.5 Host Ranges of C Type Retroviruses

Murine C-type retroviruses can be classified on the basis of their potential host range encoded in the env gene (Coffin, 1990). In other words, the env gene determines which cell type a particular virus can infect. Those viruses isolated from laboratory strains fall into two main groups, ecotropic and non-ecotropic. The ecotropic MuLVs are infectious only in the mouse, can be horizontally and vertically transmitted, and can therefore be both exogenous and endogenous. The larger group of non-ecotropic viruses comprise a set of closely related viruses, which can be distinguished by the use of specific oligonucleotide hybridization probes (Stoye and Coffin, 1987; Stoye and Coffin, 1988). The first of these are the xenotropic viruses, which are also found as endogenous murine viruses. They use different receptors to the ecotropic viruses and are not associated with disease. However, they can only replicate in non-mouse species (except in some wild mouse strains) due to receptor incompatibilities. Most mice contain only one or no ecotropic viruses (Jenkins et al, 1982), whereas the number of xenotropic viruses is usually higher (Stoye and Coffin, 1988). Next among the non-ecotropic viruses are a group which were first identified by their ability to cause cytopathic foci on mink cells and are thus known as mink cell focus-forming (MCF) viruses (Hartley et al, 1977). MCF viruses are thought to arise by recombination between endogenous ecotropic and non-ecotropic viruses but contain an altered env gene relative to ecotropic viruses (Khan, 1984; Stoye et al, 1991b). These viruses use receptors allelic to those of the xenotropic viruses and can replicate in both mouse and non-mouse species; consequently they are also known as polytropic or dual tropic viruses. Based on sequence comparisons with the non-ecotropic viruses, a second group of
MCF viruses, the modified polytropic viruses, were identified (Stoye and Coffin, 1987). A final group of non-ecotropic viruses are the amphotropic retroviruses which can infect both mouse and non-mouse species. No endogenous examples have been found in inbred mice (O’Neill et al, 1987).

1.2 Aspects of the Virus Life Cycle Significant to Fv-1 Restriction

It would be useful to focus briefly upon certain events in the virus life cycle which may be especially relevant to Fv-1 restriction. These concern the exact nature of the viral DNA which integrates into the host genome, the sub-viral structures which are present and the method by which the virus can gain access to the nucleus. The role which Fv-1 might play in these situations will be discussed later.

1.2.1 Structures Which Mediate Virus Integration

An important aspect of the life cycle is to understand how all the components necessary for reverse transcription and integration are kept in proximity to one another. If the enzymes and nucleic acids required for these steps were, after entry of the virus, to be dispersed in the cell’s cytoplasm, successful completion of the cycle would be virtually impossible because of this dilution. In addition, a mechanism must exist which prevents the virus DNA from undergoing autointegration (see later). Integration of the provirus has been extensively studied (reviewed by Brown, 1990; Grandgenett and Mumm, 1990; Goff, 1992). Using a cell-free integration system it was possible to show that the structure of the reaction product is the same as that expected from an authentic MuLV integration reaction (Brown et al, 1987). It was suggested that this was possible only in the presence of a sub-viral structure which maintained a high degree of order during infection, and in which the retroviral DNA in an infected cell
was tightly associated with the enzymatic machinery required for integration. Direct evidence for such a structure became apparent when, using the cell-free integration method, it was shown that viral DNA and integration activity could be co-purified as a large (160 S) nucleoprotein complex, both from the cytoplasm and nucleus (Bowerman et al., 1989). This would avoid the dilution of essential factors in the cytoplasm and the nucleus. Analysis of this structure by immunoprecipitation revealed that the major capsid protein p30 was a part of this complex. However, the overall conformation was different to that of the extracellular capsid since the complex was recognised by only a sub-set of anti-capsid sera. It was also demonstrated that the viral DNA was susceptible to nuclease attack, indicating that this complex was probably a more "open" structure within a cell than when in its native extracellular conformation.

1.2.2 Integration of the Viral DNA into the Host Genome

Another important aspect of the viral life cycle is the form of viral DNA which takes part in the integration step. Up to three unintegrated forms of viral DNA can be found in an infected cell. The first is the linear double stranded DNA with an LTR at each end, which is the immediate product of reverse transcription. The two other forms are circular molecules with either one or two tandemly repeated LTRs. Finally, there is the integrated proviral DNA which has an LTR at either end. The circular DNAs appear in the nucleus soon after the linear DNA and are probably formed as a result of homologous recombination or ligation events between the LTRs. Because these mechanisms require nuclear enzymatic activity, circle formation can probably be seen as a marker for entry of the virus into the nucleus. Since all these unintegrated forms can apparently be found in the nucleus, it has been difficult to determine which is involved in integration.
Using an *in vitro* integration assay, it was discovered that cytoplasmic extracts of infected cells were more active than nuclear extracts for viral integration (Brown *et al.*, 1987). They found that integration activity was proportional to the amount of linear form of viral DNA in their extract, rather than the quantity of one- or two-LTR circles. In fact, no circles were found in the cytoplasm. This phenomenon was further investigated to see if the linear viral DNA integrated directly, or via an intermediate DNA structure (Fujiwara and Mizuuchi, 1988). No intermediates were found, indicating that the linear form could integrate directly, although this could not rule out completely the possibility of circular forms being able to integrate. Viral constructs were generated which contained an internal LTR-LTR junction such as is found in the 2-LTR circles which were originally thought to be the precursors of integration (Lobel *et al.*, 1989). It was found that the LTR-LTR junction was used at least 1000 fold less efficiently than the natural sequences at the ends of the genome. Studies with Moloney MuLV demonstrated the requirement for the integrase enzyme in this reaction and pointed to a specific interaction between IN and the viral DNA ends (Brown *et al.*, 1989; Roth *et al.*, 1989; Craigie *et al.*, 1990). Integration of the linear form was also confirmed. It would appear, therefore, that the circular forms of viral DNA are in fact dead-end by-products and cannot take part in integration.

The precise biochemical reaction mechanisms for integration were suggested which involved DNA cutting and joining steps (Brown *et al.*, 1989; Fujiwara and Craigie, 1989). Briefly, two bases are removed from each 3' end of the linear double-stranded viral DNA followed by the introduction of a staggered break in the target DNA and subsequent DNA strand transfer to join the recessed 3' ends to the 5' ends of nicked target DNA strands; reviewed by Goff (1992). It is integrase alone which carries out these
reactions and it has been shown that the cleavage, strand-transfer and joining reactions occur by a one-step mechanism without involvement of a protein-DNA structural intermediate (Engelman et al, 1991). After joining, host enzymes remove the unpaired bases from the 5’ ends of the viral DNA, fill in the gaps and then repair the nicks.

1.2.3 Entry of the Virus into the Nucleus

A problem is posed by the nucleoprotein complex, in that how does it pass through the nuclear membrane? One possibility, although highly unlikely considering its size, is that it enters by passive diffusion through a nuclear pore. Or perhaps, an active transport system using nuclear localization signals may be employed. A more likely alternative is that the complex enters the nucleus when the nuclear membrane is transiently disassembled during mitosis. The predicted dependence of integration on mitosis was demonstrated. By treating cells with nocodazole (a microtubule-disrupting agent) or aphidicolin, both of which arrest cells reversibly in metaphase, retroviral integration was prevented (Roe, 1993). Viral nucleoprotein complexes isolated from arrested cells contained full-length viral DNA and were integration competent in vitro. On relieving the mitotic block, nuclear membrane breakdown occurred and integration commenced. Further evidence that a virus must wait for envelope disassembly comes from the fact that only one of the daughter cells, from a single freshly infected cell, contains integrated virus (Hajihosseini et al, 1993). When the envelope breaks down and the virus can enter, DNA replication has already taken place. If the virus were to enter before mitosis, both daughter cells would inherit a copy of the virus.

The human immunodeficiency virus (HIV) however, solves the problem by the use of a nuclear localization signal encoded in the amino terminus of
the \textit{gag} matrix protein. This facilitates active nuclear transport of the pre-integration complex in non-proliferating cells (Bukrinsky \textit{et al}, 1993; Lewis \textit{et al}, 1992).

1.3 MuLV Infection and Its Control by the Host Genome

Since the discovery of the role which MuLVs play in leukaemogenesis it was apparent that the host genetic constitution played an important part in influencing the outcome of infection. There follows a brief account of the discovery of such leukaemogenic viruses, the pathology of the disease they cause and the subsequent discovery of the host genes which were involved.

1.3.1 The Discovery of the Viral Nature of Murine Leukaemias

The first indication that a virus could induce tumour formation came near the beginning of this century. While studying the cause of a spontaneous chicken sarcoma, it was discovered that cell-free filtrates from tumours were able to produce tumours in previously healthy chickens (Rous, 1911). In subsequent years, certain laboratory strains of mouse were generated which also had a high incidence of spontaneous leukaemias. It had been assumed that only genetic factors played a role in the cause, susceptibility and development of leukaemia. A viral cause was suspected, however, it was not until 1951 that the existence of just such a leukaemogenic virus from these strains was demonstrated (Gross, 1951).

Gross found that leukaemia could be induced in low-leukaemic C3H mice by infecting new-born mice with cell-free extracts obtained from the organs of high-leukaemic AKR mice. High leukaemic strains develop an 80-90\% incidence of spontaneous thymic lymphoma between six and twelve months of age (Mayer \textit{et al}, 1978). After day 16 of gestation, these
mice begin to express endogenous ecotropic MuLV at high levels in many tissues (Rowe and Pincus, 1972). The disease is a thymus-dependent lymphoma originating in the thymus at about six to twelve months and progresses to disease involving the spleen, liver, lymph nodes and sometimes the peripheral blood in late disease (Lilly and Pincus, 1973). Inheritance of endogenous ecotropic proviruses is one prerequisite to disease. However, in AKR mice, the actual leukaemogenic agent is a recombinant MCF virus generated following a complex pathway of events (Stoye et al, 1991b). Integration of the MCF virus leads to activation of a number of cellular oncogenes (Nusse and Berns, 1988).

Acceptance of the existence of a leukaemogenic virus was not immediately universal, since some mouse strains could not be infected. It was, however, eventually realised that this was due, in part, to host range restrictions which existed, i.e., the genetic makeup of the mouse could influence the outcome of infection.

1.3.2 A Second Group of Leukaemia Viruses and Their Pathology
In 1957, a second group of leukaemia viruses was identified from cell-free extracts of leukaemic Swiss mice (Friend, 1957). In contrast to the Gross leukaemia virus, which induced a chronic leukaemia with a relatively long latency period, Friend virus (FV) could induce disease in adult mice, as opposed to just in new-born mice, after only a short period of incubation, i.e., Friend virus was an acutely transforming virus. The disease was characterized by marked proliferation of immature mononuclear cells which invaded the spleen, liver, bone marrow, kidney, lung and appeared in the peripheral blood. After two to three weeks post infection, mice would develop enlarged spleens, followed by death after one to three months. The terminal stages of the disease were characterized by greatly elevated white cell blood counts, anaemia, and highly enlarged spleen and liver. Death
would usually be caused by splenic rupture (Friend, 1957; Teich et al, 1984). In contrast also to Gross virus induced disease, Friend virus disease appeared not to involve the thymus or lymph nodes, but predominantly involved the spleen, bone marrow and later the liver. Since the major cell type involved was an erythroid precursor, this disease was referred to as an erythroleukaemia (Lilly and Pincus, 1973). A number of murine leukaemia viruses have also been shown to induce central nervous system pathology when inoculated into susceptible strains of neonatal mice. These MuLVs can infect neuronal, glial and endothelial cells (Park et al, 1993). However, in common with Gross virus, the pathogenicity of Friend virus was again restricted to certain strains of mice, with Swiss and DBA/2 strains found to be most susceptible, whereas C57BL strains were observed to be resistant. Another important factor was also noted; susceptibility to the Friend virus was not necessarily an all-or-none phenomenon. Depending on the virus-mouse combination, varying degrees of susceptibility and resistance could be observed. Once more this pointed towards genetic factors which could influence leukaemogenesis.

1.3.3 The Complex Nature of Friend Virus Disease

How Friend virus induces such rapid erythroleukaemia in mice is not clear, however work on the erythropoietin receptor has suggested a possible mechanism (see section 1.4.4). In other leukaemic viruses, several methods exist whereby they can cause pathogenesis. Some viruses contain non-virus related sequences (oncogenes) which initiate transformation. Others cause insertional activation of oncogenes in cis or activation of proto-oncogenes in trans often through a multi-step process and by expressed viral gene products which act as transcription factors (Varmus and Brown, 1989). The insertion of a retrovirus lacking oncogenes may bring proto-oncogenes under the transcriptional control of the LTR.
During subsequent studies of Friend virus disease, it was observed that different derivatives of the original virus produced different manifestations of the disease. One preparation would induce rapid splenomegaly, with marked increase in levels of red cells in the peripheral blood (polycythaemia, hence FV-P). Other preparations would, instead, induce varying degrees of anaemia (hence FV-A), as was the case in the first thymus-independent disease described (Friend, 1957).

Following the development of an *in vivo* spleen focus assay for Friend virus it was shown that FV-P gave high titres in the assay, whereas FV-A gave far lower, if any, titres (Mirand *et al*, 1968). The separable component of Friend virus responsible for focus formation was thus termed SFFVp or SFFVA (for spleen focus forming virus). It had also been found that Friend virus could induce lymphatic leukaemia in rats, although when extracts of these leukaemias were reintroduced into mice the original erythroblastic disease reappeared. Subsequently it was demonstrated that after several serial passages of the virus through rats, the result was a virus which induced only lymphatic leukaemia in mice, with the erythroblastic disease having been lost (Lilly and Pincus, 1973). The lymphatic leukaemia component of Friend virus was similar to that found in the original virus isolated by Gross, neither of which had the capacity to form spleen foci.

It thus appears that the lymphatic component and the SFFV component were separable and that together they formed the Friend virus complex. The relationship between the two is that of helper virus and replication-defective virus. The replication-defective viruses, in this case SFFVA and SFFVp, have been cloned and sequenced and were found to lack a portion of the MuLV genome involved in envelope synthesis, so blocking the replication cycle (van Beveren *et al*, 1985). SFFV must therefore be “rescued”
by a helper virus replicating in the same cell, in this case, Friend MuLV, which supplies SFFV with the missing proteins and thereby allows the SFFV replication cycle to by-pass the block. The oncogenicity of the SFFV virus is a result of the altered \textit{env} sequence (see section 1.4.4), rather than being due to the acquisition of a new sequence (Coffin, 1984).

So, it is the defective SFFV component which causes the acute oncogenic, short-latency disease and the Friend MuLV component which gives rise to the long-latency, chronic, thymus-dependent leukaemia.

1.4 Genetic Determinants Governing Susceptibility to Friend Virus

It quickly became apparent from the first studies with Friend virus that host genetic factors could influence the outcome of infection since viral isolates could be transmitted to some mouse strains but not others.

1.4.1 The Discovery of \textit{Fv-1} (and \textit{Fv-2})

In 1962, the first investigation of a genetic factor which could influence the susceptibility to Friend virus was carried out (Odaka and Yamamoto, 1962). By analysing crosses of a resistant strain (C57BL/6) with a susceptible strain (RF), it was concluded that a single autosomal gene, with two alleles, controlled susceptibility and resistance and that the allele for susceptibility was dominant over the allele for resistance. This gene was given the symbol \textit{Fv}, with dominant allele \textit{Fv}^s (for Friend virus susceptibility) and resistance allele \textit{Fv}^r (for resistance; Odaka, 1969). Later, however, results emerged which indicated that the picture was more complex that had previously been thought. Using the spleen focus assay (Axelrad and Steeves, 1964) it was found that F1 hybrids of resistant C57BL/6 (\textit{Fv}^r/r) x susceptible DBA/2 (\textit{Fv}^s/s) showed an intermediate susceptibility. This was also true for Balb/c
mice although these are supposedly homozygous at all autosomal loci (Lilly, 1967). There was now a possibility that more than one gene was involved in Friend virus response.

The problem was resolved using two isolates of Friend virus (the original F-S and a variant, F-B) to investigate the inheritance of susceptibility as measured by the spleen focus-forming property of Friend virus (Lilly, 1970). Two independently segregating genes were shown to be responsible for the complex pattern of inheritance of susceptibility to Friend virus. The genes were named Fv-1 and Fv-2. Fv-1 controlled relative resistance to F-S, where the allele for resistance was dominant (Fv-1\(^r\)) and susceptibility (Fv-1\(^s\)) was recessive. This gene did not, however, influence the outcome of infection with F-B virus. Fv-2 governed absolute resistance to focus formation by both F-S and F-B viruses, at which the allele for susceptibility was dominant. Fv-2 was also shown to be linked to the dilute locus on chromosome 9, but was not linked to Fv-1. So, it emerged that Fv-2 appeared to act on the SFFV component of Friend virus, since homozygosity for the resistance allele (Fv-2\(^r\)) caused the host to be resistant to focus formation by SFFV, but this did not affect the lymphatic leukaemia component (the helper virus) which could be isolated from Fv-2\(^r\) mice under certain conditions (Lilly, 1973 #38). In retrospect, the first studies by Odaka appear to have identified Fv-2, and not Fv-1.

1.4.2 The Alleles of Fv-1

In separate work, a tissue culture assay, developed to study viral replication, was used to examine MuLV replication in various cell types (Hartley et al, 1970). Different laboratory and field virus isolates were used and it was found that all viruses fell into one of three categories with respect to their ability to propagate in cells of NIH Swiss and Balb/c mouse embryo cultures. One group of viruses replicated 100-1000 fold better in NIH cells than in
Balb/c and were thus named N-tropic. A second group of viruses showed
the opposite pattern of infection and were named B-tropic. A third category
of virus replicated equally well in both cell types and were named NB-
tropic. In a subsequent series of studies, the tissue culture assay was used to
show that a single genetic locus, with two alleles, was the major
determinant in the mouse cells for in vitro infection with MuLV (Pincus et
al, 1971a). All cells tested resembled either NIH Swiss or Balb/c with respect
to virus replication, i.e., they were either N-type or B-type. It was possible to
show that resistance was dominant in regard to both N- and B-tropic virus
(co-dominant alleles). It became apparent that this genetic locus was the
same, or closely linked to the gene which had already been described for
resistance to Friend virus infection, namely Fv-1 (Pincus et al, 1971b). Fv-2,
however, which had been earlier found to govern sensitivity to spleen-
focus induction in vivo by both F-S and F-B variants of Friend MuLV, did
not exert an effect in vitro. This indicated that perhaps the fibroblasts used
did not express Fv-2, which was most likely a property of the
haematopoietic target cells of the virus (see section 1.4.4). In addition, the
presence or absence of the SFFV component of Friend virus showed no
detectable influence upon the assay, which supported the view that Fv-2
affected the SFFV component. It was therefore only Fv-1 which influenced
the in vitro assay by acting on the helper component of the virus (Lilly and
Pincus, 1973). The assay showed that F-S virus was N-tropic and that F-B
virus was NB-tropic, which explained the fact that Fv-1 had no influence
upon the outcome of F-B virus infection (Lilly, 1970). The Fv-1 gene system
had first been described using the N-tropic F-S virus, giving rise to the gene
symbols Fv-1^ for resistance and Fv-1^ for sensitivity. However, now that
Fv-1 was known to have co-dominant resistance alleles, they were renamed
Fv-1^ (resistant to N-tropic virus, but susceptible to B-tropic virus) and Fv-1^ (resistant to B-tropic virus, but susceptible to N-tropic virus) respectively.
Since the \( Fv-1 \) phenotype could be detected in both mice and cultured cells, this suggested that the resistance to infection had to be on a cellular level and could not involve mechanisms requiring complex cellular interactions, immunological or other hormonal pathways (Jolicoeur, 1979).

1.4.3 Other Alleles of \( Fv-1 \)

\( Fv-1^{nr} \): A third allele of \( Fv-1, Fv-1^{nr} \) has been reported (Steeves and Lilly, 1977) in strains 129, RF/J, RFM/Un, NZB and NZW. These had previously been characterized as \( Fv-1^{n} \) (susceptible to N-tropic virus) but were in fact ten-fold more resistant to some endogenous N-tropic MuLVs than other \( Fv-1^{n} \) strains. In crosses between CBA (\( Fv-1^{n} \)) and 129 mice it was found that \( Fv-1^{nr} \) was dominant over \( Fv-1^{n} \). Further evidence for another allele came from studies in crosses between the high leukaemic AKR/J mouse strain and RF/J mice. Animals were typed by preparing spleen and thymus extracts and using these to infect susceptible SC-1 cells (Hartley and Rowe, 1975) followed by the XC plaque assay (Rowe et al, 1970). Both strains were found to be of the \( Fv-1^{n} \) type, i.e., resistant to B-tropic virus. However, they were found to differ in their response to AKR-derived N-ecotropic MuLV infection (Mayer et al, 1978). The \( Fv-1^{nRF} \) allele was found to reduce the expression of the N-tropic MuLV when compared to \( Fv-1^{nAKR} \). This was observed when the RF strain and (AKR x RF)F1 hybrid mice displayed a much lower and later lymphoma incidence than did AKR parental mice. The same was also true in their effect on xenotropic MuLV expression and lymphoma development. The conclusion was that the \( Fv-1^{n} \) type represented a category of alleles rather than a single allele. A sub group of N-tropic viruses are not restricted by \( Fv-1^{nr} \) hosts; these are called N\( r \)-tropic (Heitman et al, 1991).
However, the $Fv-1^{nr}$ phenotype may not be encoded by $Fv-1$. Investigations into the phenotype of embryonal carcinoma (EC) cell lines derived from the 129 strain ($Fv-1^{nr}$) found that one of these (F9 cells) were apparently $Fv-1^a$, i.e., more permissive for most N-tropic viruses than $Fv-1^{nr}$ (Heitman et al, 1991). Another line (PCC4.aza1R) was found to be phenotypically silent for $Fv-1$ expression. Furthermore, some differentiated derivatives of PCC4.aza1R did restrict viral replication and were found to be $Fv-1^{nr}$. In other words, different cell lines derived from the same mouse strain were differentially expressing $Fv-1^a$ and $Fv-1^{nr}$ alleles. Unpublished results from crosses of Balb/c ($Fv-1^b$) and RFM/Un ($Fv-1^{nr}$) suggested that the $Fv-1^{nr}$ allele segregated independently of the $Fv-1^b$ locus (Yang W.K. et al; cited in Heitman et al, 1991). The experiments with F9 cells, because they restricted B-tropic virus but no longer displayed the $Fv-1^{nr}$ phenotype, also suggested that these two alleles were separable. Alternatively, the $Fv-1^{nr}$ phenotype could be the result of a separate gene(s) which is not expressed in F9 cells, but modifies normal $Fv-1^a$ function. The $Fv-1^{nr}$ phenotype was therefore not actually due to an $Fv-1$ allele. The viral tropism of this additional restriction does appear, however, to map to the same region of the viral genome (Boone L.R. cited in Heitman et al, 1991; see section 1.6.1).

$Fv-1^o$: analysis of wild mice has revealed another allele (Kozak, 1985). For example, Mus spretus and Mus musculus praetextus do not restrict either N- or B-tropic MuLV. Genetic analysis demonstrated that this non-resistance phenotype was controlled by the novel allele $Fv-1^o$.

$Fv-1^{null}$: Some other cell lines appear to have lost $Fv-1$ restriction. One such is the 3T3FL line, which originated from a Swiss mouse embryo (Todaro and Green, 1963). The line was originally designated as $Fv-1^b$ (Todaro, 1972). It was demonstrated that 3T3FL cells were susceptible to
virus of either tropism, which suggested that $Fv$-$1$ was either missing or not expressed (Krontiris et al, 1973; Gisselbrecht et al, 1974). A further cell line called SC-1 (Hartley and Rowe, 1975) likewise showed particular sensitivity to N- and B-tropic viruses. This line had, however, been derived from a $Fv$-$1^-$ embryo culture.

1.4.4 Other $Fv$ Genes

In addition to $Fv$-$1$, mice posses other genetic loci which affect viral replication and spread. It would be useful to set $Fv$-$1$ in the context of these restriction genes so they will be outlined briefly.

$Fv$-$2$

The discovery of $Fv$-$2$ and its control over SFFV pathogenicity has already been discussed, but the normal function of $Fv$-$2$ in the absence of infection remains unresolved. A function for the gene product in relation to viral restriction has now been suggested (Axelrad et al, 1981). It was shown that the removal, by washing, of a molecule normally present in erythropoietic progenitor cells (BUF-E) in mice of the $Fv$-$2^{r/r}$ genotype, resulted in a dramatic increase in DNA synthesis. These results were confirmed (Suzuki and Axelrad, 1980) and extended to show that the effect was restricted to only this particular cell type. This work implied the presence of a negative growth control mechanism in these cells. The washable extract, when applied to $Fv$-$2^{s/s}$ cells, induced the negative growth regulation which was characteristic of $Fv$-$2^{r/r}$ cells. It was postulated that $Fv$-$2^r$ was the negative growth regulator acting only in the absence of the $Fv$-$2^s$ product. Several years later it was demonstrated that the gp55 envelope glycoprotein of SFFV associated with erythropoietin receptors (EpoR) to cause inappropriate proliferation of infected erythroblasts (Li et al, 1990). In other words, gp55 mimicked the action of erythropoietin by binding to its receptor. This effect
was also independently demonstrated (Hoatlin et al, 1990), and it was also determined that the Fv-2 gene did not encode EpoR (in fact both genes map to chromosome 9 but on opposite ends). It was suggested that Fv-2 down-regulated EpoR signal transduction. Interestingly it was noted that the down-regulation signal for the BUF-E erythropoietic progenitor cells in Fv-2/2 r strains did not cause erythrocyte insufficiency in uninfected mice maintained in normal conditions. So, in Fv-2/2 r mice the proliferative effect of gp55 is blocked. However, a Friend virus mutant was obtained with a truncated envelope protein that overcame this block and could specifically bind to and activate EpoR (Kozak et al, 1993). A model was proposed in which the Fv-2 r-encoded protein could associate with EpoR to control its activation by various ligands including envelope glycoproteins and erythropoietin. The Fv-2 r protein could perhaps no longer interfere with and prevent the action of the mutant envelope ligand. This model is compatible with the down-regulation of DNA synthesis by the Fv-2 r factor (Axelrad et al, 1981).

Fv-4

A new locus was first identified in the non-inbred mouse strain G (Suzuki, 1975) which restricted N-, B- and NB-tropic viruses, and prevented the development of splenomegaly by Friend virus (Kai et al 1976 in Teich et al, 1984). Identity with Fv-1 or Fv-2 was excluded since the gene segregated independently of these. The gene was termed Fv-4. (the name Fv-3 had been provisionally used by Odaka, 1973) but was abandoned because of its possible identity with Fv-1). The allele for resistance (Fv-4 r) was dominant over susceptibility (Fv-4 s).

A gene with similar properties to Fv-4 was described (Gardner et al, 1991; Gardner et al, 1980) which was characterized in a wild mouse population. F1
hybrids of this Lake Casitas (LC) strain and the high-leukaemic strain AKR, revealed that this gene prevented viraemia and virus mediated lymphoma. The gene was named Akvr-1 (for AKR virus restriction). Later, Fv-4 and Akvr-1 were shown to be identical alleles of a single locus (O'Brien et al, 1983) on chromosome 12 (Ikeda et al, 1981). A mechanism for Fv-4 restriction became apparent when it was revealed that resistant Fv-4\(^r\) lymphocytes had a novel retroviral envelope glycoprotein on their surface, while Fv-4\(^s\) cells did not (Ikeda and Odaka, 1983; Kozak and Silver, 1985). Immunological characterization showed that gp70 was detectable on cells of the thymus, spleen, lymph nodes, bone marrow and embryo fibroblasts of Fv-4\(^r\) animals and it was suggested this could be expressed by a provirus and thus lead to viral interference (Ikeda and Odaka, 1984). A truncated ecotropic MuLV provirus was indeed discovered at the Fv-4 locus (Kozak et al, 1984), consisting of ~850 bp of the 3' end of the pol gene, a complete env gene and a 3' LTR. It was found that all Fv-4\(^r\) mice had the same piece of viral DNA which was always associated with the same flanking host DNA (Ikeda and Odaka, 1984). Fv-4 thus appears, as suggested earlier, to act by interference, i.e., the gene product (an envelope glycoprotein) blocks infection by competing with exogenous virus for cell surface receptors. The Fv-4 gene confers resistance to all ecotropic viruses including all N-, B- and NB-tropic MuLVs (Kai et al 1976 in Teich et al, 1984). Clearly, the Fv-4 gene affects more MuLVs of differing host ranges than Fv-1 does and hence masks the Fv-1 phenotype in G mice (Steeves and Lilly, 1977).

**Fv-5**

Another host gene, Fv-5, has been implicated in the alteration in erythropoietic response to Friend virus infection, i.e., whether the early erythroid response to FV-P infection is polycythaemia or anaemia. Those strains homozygous for allele Fv-5\(^s\) develop a rapid and transient anaemia,
whereas those of the $Fv-5^p$ genotype undergo polycythaemia. As with $Fv-1$, the phenotype segregates as a single co-dominant locus (Shibuya and Mak in Teich et al, 1984).

$Fv-6$

$Fv-6$ determines the susceptibility of newborn mice to erythroleukaemia on infection with the helper component of Friend virus complex. Studies with crosses between susceptible and resistant strains indicate that resistance to Friend MuLV-induced disease is dominant and segregates as a single locus. It has been suggested that the mechanism of action is mediated by an interference phenomenon that blocks either the generation or the replication of MCF virus following Friend virus MuLV infection (Ruscetti et al, 1981). This model supposes that the MCF virus plays a part in Friend MuLV disease (see section 1.3.1). MCF was shown to be important in erythroproliferative disease after inoculation of certain strains of newborn mice with Friend MuLV; furthermore focus-induction was blocked in mice expressing endogenous MCF-like viral envelope glycoprotein. The mechanism of action of $Fv-6$ may therefore be similar to that described for $Fv-4$ resistance, with an endogenous polytropic $env$ gene substituting for an ecotropic gene.

Other genes

There are several other genes including Steel (Sl), Dominant spotting (W) and flexed (f) that affect susceptibility to Friend virus induced erythroleukaemia. These genes are involved in development and differentiation, specifically haematopoietic progenitor cell function. Any abnormality in the pathways of erythropoiesis caused by alleles of these genes could interfere with erythroleukaemic induction.
1.5 The \textit{Fv-1}-Sensitive Steps in the Retroviral Life Cycle

For many years, investigators have been attempting to define or narrow down the steps in retroviral replication which are affected by \textit{Fv-1} restriction. These efforts have mainly concentrated on comparisons of the levels of the various viral RNAs and DNAs during infection of permissive and restrictive cells. Investigations have been hampered by the lack of specific virus probes and a poor understanding of the viral life cycle.

1.5.1 The Effect of \textit{Fv-1} Restriction on Virus Cell Entry

One of the first experiments which helped to narrow down the \textit{Fv-1} restriction step was the investigation of the role of the viral envelope (Huang \textit{et al}, 1973; Krontiris \textit{et al}, 1973). Firstly, the level of adsorption of Friend MuLV to susceptible and resistant cells was determined by incubating cells with virus and, after certain time intervals, removing the infection medium and measuring the virus titre remaining in the medium. It was clearly shown that virus of either N or B tropism adsorbed equally well to either cell type and that there were no significant differences in the rates of viral uptake. Then, pseudotype viruses were constructed containing vesicular stomatitis virus (VSV) genomes coated by envelopes from N- or B- tropic MuLV. Using these to infect cells, it was shown that VSV of both types replicated equally well on either restrictive or permissive embryo culture cells. This led the investigators to the conclusion that Friend MuLV envelope proteins were not involved in the host-restriction mechanism and that restriction must be an intracellular event. These results were confirmed by infecting \textit{Fv-1}^n and \textit{Fv-1}^b cells with virus of either tropism which had radioactively labelled RNA (Sveda \textit{et al}, 1974). It was found that equal amounts of labelled RNA recovered from both restrictive and permissive cells. This technique was designed to measure virus genome entry into the cell only and could not monitor viral mRNA synthesis. This
result, therefore, showed that virus could indeed enter a restrictive cell unimpeded.

1.5.2 Levels of Viral Nucleic Acids in Restrictive Infections

Subsequently, investigations turned towards the levels of the various viral nucleic acids and enzyme activity in restrictive cells infected with MuLV. Reverse transcriptase levels were measured as a direct indication of virus production, and were found to be 70-100 fold lower in restrictive cells than in permissive cells (Jolicoeur and Baltimore, 1976b). The same was true of levels of newly transcribed viral RNA in the cytoplasm and the nucleus. The conclusion was that the $Fv-1$ gene product could be either degrading viral RNA transcripts, preventing RNA transcription or causing interference with a stage before viral transcription even started, for example at viral DNA synthesis or at integration of the provirus. Proviral DNA formation was then analysed (Jolicoeur and Baltimore, 1976a). Unintegrated viral DNA from infected cells was extracted using a technique that efficiently separates low-molecular weight DNA (Hirt supernatant; Hirt, 1967) from high molecular weight DNA (pellet). DNA levels were quantified by solution hybridization to radioactively labelled MuLV DNA. No restriction of viral DNA accumulation was observed using either N- or B-tropic MuLV in permissive or restrictive cells. Integration of virus-specific DNA into chromosomal cellular DNA was also assayed and results showed that integration of proviral DNA could be detected in permissive cells but not in restrictive cells.

Up to this point, there was no consensus as to the fate of viral DNA. Other workers had previously suggested that $Fv-1$ did not prevent integration, based on evidence following the fate of input viral genomic RNA (Sveda et al, 1974). This would have suggested that $Fv-1$ restriction was a post-
integration event. However, using similar methodology to that used earlier (Jolicoeur and Baltimore, 1976a), the same investigators found that their initial results were not correct and that integration was indeed inhibited by Fv-1 (Sveda and Soeiro, 1976). The conclusion was that Fv-1 acted on the virus life cycle between synthesis of viral DNA and its integration. This supported the RNA expression studies which placed the block at a point prior to transcription.

Further investigations were carried out to confirm that Fv-1 restriction was not a late event, i.e., post-integration (Hsu et al, 1978). Total DNA was isolated from permissive SC-1 cells infected with N- or B-tropic virus. Both Fv-1" and Fv-1" cells were then transfected with the DNA preparations. Results revealed that in contrast to normal virion infection, virus production from restrictive cells was not inhibited using the transfected DNA. In other words, Fv-1 appeared not to affect transcription, translation or virus assembly and budding. This conclusion was also reached by microinjecting unintegrated viral DNA into restrictive cells and showing that this could produce a successful infection (Chinsky et al, 1984). No conclusion could be drawn about the integration step, since the virus preparations were mostly in the integrated form flanked by adjacent cellular DNA sequences. These results did, however, highlight the difference between "normal" infection and transfection. The conclusion from this work was that in order for the Fv-1 gene product to recognise and interfere with the early events in the virus life cycle, the viral DNA must be in its "native" cytoplasmic form, i.e., complexed to other viral structures.

Further work to define the point in the life cycle which is restricted was carried out (Duran-Troise et al, 1981). An amphotropic N-tropic MuLV was used to infect resistant Balb-3T3 cells and N-tropic virus-producing cloned
cells were isolated and reinfected with ecotropic MuLV. It was demonstrated that \textit{Fv-1} was still being expressed, as shown by restriction of the new virus. Examination of virus production by the amphotropic virus infected clones also showed that \textit{Fv-1} could have no effect on the expression of virus once it was integrated, therefore restriction must occur before integration takes place.

1.5.3 The Effects of \textit{Fv-1} on Linear and Circular Viral DNA

The effect of \textit{Fv-1} restriction was further investigated by comparing linear and circular forms of viral DNA in the infected cell, at the early stages of infection (Jolicoeur and Rassart, 1980; Yang \textit{et al}, 1980). At the time these experiments were carried out, it was thought that the precursor to viral integration was the 2-LTR circle and not the linear form as is now known to be the case. It was observed that, as before, levels of linear double stranded viral DNA were not significantly reduced in restrictive cells. However, closed circular DNA (both 1 and 2-LTRs) molecules, which appear after linear DNA, were reduced by 25-50 fold in resistant cells. Several possible mechanisms of \textit{Fv-1} action were suggested: 1) \textit{Fv-1} prevented the formation of circular molecules from the linear precursor. 2) The circular form was being selectively degraded. 3) The transport of linear viral DNA to the nucleus was inhibited, thereby preventing circular form accumulation which only occurred in the nucleus. 4) The double stranded linear DNA was damaged or defective as a result of \textit{Fv-1} action.

Which ever was the case, it was proposed that restriction occurred before the integration event itself. Although it was assumed to be the circular form was the integration precursor it was impossible to be certain of a particular model or whether the block in circle formation was a primary or secondary effect of \textit{Fv-1} restriction.
The questions were examined in more detail using pulse-chase experiments (Jolicoeur and Rassart, 1981). The levels of linear viral DNA in permissive and restrictive cells were found to decrease at the same rate in both cases, indicating that linear viral DNA was not being specifically degraded by the Fv-1 gene product. In permissive cells, levels of circular DNAs increased relative to levels of linear DNA, however, as expected from the earlier work, no circular forms could be detected in resistant cells. To test the hypothesis that there could be a block in nuclear transport of linear DNA into the nucleus, cells were fractionated into cytoplasmic and nuclear components. Levels of viral DNA were detected by Southern analysis. Levels of linear viral DNA were similar in both fractions of both permissive and restrictive cells. The circular form was found in the nuclear fraction of permissive cells, but could not be detected in the nucleus of resistant cells. With further pulse-chase experiments, it was shown that linear DNA accumulated in nuclei of permissive and restrictive cells (Jolicoeur and Rassart, 1981). However, as expected, circular DNA accumulated only in permissive cells. The conclusion was that Fv-1 did not interfere with nuclear transport of linear viral DNA and that no degradation of this DNA was seen in restrictive cells. It was still assumed at that time that the circular form was the precursor to integration, so the conclusion drawn from these experiments was that Fv-1 must be preventing the linear DNA from forming the pre-integration circles and that this was the cause of the reduced levels of integrated provirus.

The above results were confirmed with respect to circular DNA formation (Chinsky and Soeiro, 1981). However, contradictions arose, in that levels of linear viral DNA also appeared to drop when B-tropic MuLV was used to infect NIH (Fv-1n) cells. It was suggested that this effect might be due to another unidentified gene which modifies the Fv-1 effect, thus allowing it
to restrict the virus at several points in the early stages of infection, but this could not be substantiated. Interestingly, although levels of circular DNA were observed to drop in restrictive cells, the reduction was only in the order of 2-3 fold when compared with permissive cells. This did not correlate quantitatively with the reduction of infectious virus recovered and was clearly different from the 25-50 fold reduction in circle formation observed in the earlier experiment (Jolicoeur and Rassart, 1980) which showed good correlation with fall in virus production.

The data generated by all these investigations seems to present a rather hazy picture of the point in the retroviral life cycle when Fv-1 restriction may act. On balance, it seems likely that restriction occurs between viral DNA entry into the nucleus and the integration of the provirus into the host genome. Whether the restrictive step is at or before integration remains to be discovered. The work by Brown et al (1987) and the other groups which helped to demonstrate that the linear DNA, not the circular DNA was the molecule which integrated, does not necessarily help clarify the puzzle. It has been shown that the cytoplasm (where only the linear form is to be found) is a better source of active proviral precursors than the nucleus (where the circles are found), yet it is the circular form, not the linear form, which is reduced in restrictive cells. This does pose a problem in the understanding of Fv-1 restriction. The reduced circle formation is surely significant, even if it is merely a side-effect of the restriction mechanism.

A recent reinvestigation of the problem using different techniques and with a better understanding of the biochemistry of integration has been carried out (Pryciak and Varmus, 1992). Unfortunately, this has shed no further light on this problem. It was observed that in restrictive cells, cytoplasmic levels of viral DNA were reduced, a decrease which correlated well with the
degree of restriction. However, no difference in the levels of linear DNA in the nucleus was observed, and viral DNA appeared fully competent in *in vitro* integration assays. There is no simple explanation for these contradictory data.

1.6 How Does *Fv-1* Interact With the Virus?

The question as to exactly when *Fv-1* exerts its effect in the virus life cycle remains to be answered. The question as to how the *Fv-1* gene product might interact physically with the virus has also been addressed.

1.6.1 The *Fv-1* Tropism Determinant

It was known that ecotropic MuLVs could be classified according to their host range as either N-, B- or NB-tropic and that this phenotype was determined by *Fv-1* (Hartley et al., 1970). Virus which has been force-passaged through resistant cell lines can be converted to NB-tropism, i.e., the virus can replicate equally well in both *Fv-1*<sup>n</sup> and *Fv-1*<sup>b</sup> cells. The electrophoretic mobility on SDS polyacrylamide gels of three virion proteins of B- and NB-tropic viruses was investigated (Hopkins et al., 1977; Schindler et al., 1977). The three protein were p15 (matrix), p30 (capsid) and gp70 (envelope glycoprotein). In this experiment, proteins of B-tropic virus from Balb/c mice and six NB-tropic viruses derived from the B-tropic virus by serial passage through NIH cells, were used. The results revealed that while p15 and gp70 remained the same in B- and NB-tropic preparations, p30 from NB-tropic virus migrated more rapidly than p30 from B-tropic virus. Whether this change was directly responsible for altered tropism was not clear. The involvement of a virus protein in the determination of tropism had also been suggested by earlier work (Bassin et al., 1975) where murine sarcoma virus pseudotypes, which had been rescued by MuLV, acquired a
determinant specifying N or B tropism characteristic of the helper virus. This implied that the determinant was not viral RNA but was rather a protein product encoded by it. This was confirmed by work in which a helper virus-free Fv-1-sensitive packaging system was developed (Boone et al, 1989). By definition, no viral RNA was present, therefore a viral protein was the determinant.

Peptide maps were constructed of the p30 protein of more than 50 isolates of MuLV and showed that, in general, the primary structure of the protein was highly conserved, in contrast to a high degree of polymorphism found in gp70 (Gautsch et al, 1978). However, a small structurally variable region of p30 was identified involving only a single peptide which correlated precisely with the Fv-1 tropism of the viral isolate and could therefore be used to reliably classify virus from any source. This finding supported the previous biochemical and genetic data suggesting that p30 was the viral determinant of Fv-1 tropism (Hopkins et al, 1977; Schindler et al, 1977). Using RNase T1 fingerprinting of the genomes of viruses (Faller and Hopkins, 1977; Faller and Hopkins, 1978) three large RNase-resistant oligonucleotides were identified, each of which was associated with either N-, B- or NB-tropism and which were located in the 5' coding region of the virus (in the p30 region). These oligonucleotides were sequenced (Rommelaere et al, 1979) and it was shown that they shared certain sequences indicating that they were allelic and that certain nucleotide changes were characteristic of particular viral tropisms. Although p30 was now implicated in Fv-1 tropism, its biochemical function was unknown. At that time it was not even possible to determine the location of these sequences in the p30 coding sequence.
Recombinant DNA techniques were used to locate the Fv-1 determinants (Boone et al, 1983). Permissive SC-1 cells were infected with either N- or B-tropic virus, circular DNA was recovered, the viral DNAs digested at a conserved HindIII site and cloned into pBR vectors. Precisely defined restriction fragments were then exchanged between the two clones. Infectious virus was recovered and assayed in Fv-1\(^n\) and Fv-1\(^b\) cells. The results revealed that the determinant for Fv-1 was encoded by a 1.3 kb BamHI to HindIII fragment extending from ~120 bp into the p30 coding region up to ~380 bp inside the pol gene. Almost identical experiments were carried out independently (DesGroseillers and Jolicoeur, 1983), and the determinant was narrowed down to a 302 bp BamHI-BstXI 5' end fragment of p30. The sequence of this fragment confirmed that it coded for p30 and showed that two consecutive amino acids, at codons 109 and 110, together or singly, defined viral tropism. In the case of N-tropic virus, the sequence was glutamine-arginine and for B-tropic virus it was threonine-glutamic acid. The 1.3 kb BamHI-HindIII fragment was also sequenced, confirming the amino acid variations at codons 109 and 110 (Ou et al, 1983).

These changes, on the basis of their charge, explain the previously described electrophoretic properties of p30 (Hopkins et al, 1977; Schindler et al, 1977). Two different NB-tropic isolates have glutamine at position 109 but different amino acids at 110. However, modifications both up stream and down stream were also present. The involvement of other codons in NB-tropism was demonstrated by work in which the NB-tropic Moloney MuLV was modified by site-directed mutagenesis (Pryciak and Varmus, 1992). Codons 109 and 110 (glutamine and alanine) of MoMuLV were changed either to the N- or the B-tropic codons. These mutations, however, did not convert the virus to N- or B- tropism.
The relationship between N and B tropic determinants and their relationship to other endogenous non-ecotropic MuLVs was analysed (Boone et al, 1988). This study suggested that the B-tropic determinant in B-tropic ecotropic MuLVs may have arisen from recombination between N-tropic ecotropic MuLVs and members of the abundant endogenous non-ecotropic proviruses.

1.6.2 Interaction of Fv-1 With the Pre-Integration Complex

The Fv-1 gene product must somehow physically interact with p30. Initially it was unclear where this interaction took place. It was widely believed that complete dissociation of the viral RNA and its capsid took place following entry into the cell. How then could a p30-Fv-1 gene product interaction affect integration? The later discovery of an integration-competent nucleoprotein complex derived from the virion particle, provided a solution to this problem. A means was suggested by the fact that integration-competent nucleoprotein complex could be isolated from infected cells. This sub-viral structure appeared to be in a relatively open or relaxed conformation which was different to that of the core structure found in the extracellular virus. Most importantly, it was shown to contain the capsid protein p30 (Bowerman et al, 1989). Therefore the Fv-1 determinant is present and associated with viral DNA intracellularly and is presumably "visible" to the Fv-1 gene product. This implies that the Fv-1 gene product interacts directly with the nucleoprotein complex preventing integration from taking place.

The question of an interaction in restrictive cells with the nucleoprotein complex has been investigated (Pryciak and Varmus, 1992). Using an in vitro integration assay, it was demonstrated that viral complexes from nuclei isolated from restrictive infections were just as competent to
integrate as those from permissive infections. This meant that the pre-integration complexes were not irreversibly damaged or affected by \textit{Fv-1}, for example by proteolytic cleavage or disassembly. It was suggested that during preparation of the extracts, the restrictive component might become dissociated and could therefore no longer exert its effect \textit{in vitro}. Alternatively, \textit{in vitro} integration could bypass the block by following a slightly different integration mechanism to that \textit{in vivo}. Significantly, it was also found that the integrase-dependent trimming of the 3' ends of the viral DNA necessary for integration, occurred normally in restrictive cells. This suggested that \textit{Fv-1} was not interfering with the enzymatic machinery of integration. However, it could still be argued that the block was at a later stage.

1.7 Kinetics of Virus Infection in Permissive and Restrictive Cells

A further aspect of virus replication and its inhibition by \textit{Fv-1} is the amount of virus which is needed to overcome restriction in order to establish a successful infection. Many groups have studied the dose response curves and the kinetics of permissive and restrictive infections. This work has been exhaustively reviewed by Jolicoeur (1979). It is known that if cells are inoculated at a high multiplicity of infection (MOI) that restriction can be overcome. It was found that resistant cells showed two-hit kinetics, while titrations in permissive cells gave a one-hit curve (Declèве \textit{et al}, 1975; Pincus \textit{et al}, 1975). This phenomenon was termed the "hitness factor" and was interpreted by supposing that one virus could not overcome the block to infection, but required a second virus. It was first reported that restriction could be overcome if a cell was co-infected with UV-inactivated MuLV of the same tropism (Yoshikura, 1973). Apart from revealing two-hit kinetics, this result also suggested that \textit{Fv-1} must act via the interaction with viral
structures other than its nucleic acids. The two-hit kinetics was confirmed, (Duran-Troise et al, 1977) and it was calculated that two MuLV particles were required to successfully infect resistant Balb/3T3 cells with N-tropic virus. The second particle had to enter the cell within ~3 hours of the first. The effect of the first particle knocking out Fv-1 restriction, and making way for a second particle to initiate a productive infection, was termed “abrogation”. There was no apparent need for the first virus to replicate for abrogation to be effective. It has also been shown that genome-deficient virions were able to abrogate Fv-1 restriction, which allowed a second particle to establish an infection (Boone et al, 1990). This once again, showed that Fv-1 restriction was active in the absence of any viral genome-directed process and that another viral structure was involved.

In contrast, other workers have reported one-hit curves in restrictive cells (Jolicoeur and Baltimore, 1975; Boone et al, 1989) implying that it is possible to infect restrictive cells without abrogating Fv-1 restriction. The reasons for this apparent contradiction are not clear. However, from previous work it does appear that it can be difficult for different laboratories to reproduce their own results, let alone each others (Jolicoeur, 1979). It seems quite likely that the experimental results are very sensitive to the strain of mice used, as well as to the strain-virus combination and the virus titre used.

On the other hand, it is not impossible that both one and two hit curves are correct. I argue that infection of a restrictive cell could take place either as a result of a stochastic leakiness in restriction, or as a result of abrogation. For example, it is possible that at a low, but constant frequency, the virus is able to evade Fv-1 restriction, and as long as the virus titre does not increase beyond a certain point, the number of infected cells will remain directly proportional to the number of virus particles added. If the input virus titre increases
beyond a certain point, then the single-hit kinetics will be replaced by two-hit kinetics, since those cells left which have not already been infected by a stochastic event will be restrictive and require abrogation of \( Fv-1 \) by one virus before another can successfully infect.

1.8 The Mechanism of \( Fv-1 \) Restriction

It is now possible to put forward possible models for the means by which \( Fv-1 \) is able to restrict the replication of MuLV. Several facts have become clear:

a) It is known that the virus can enter a restrictive cell, so the envelope which is removed upon endocytosis cannot be involved in restriction.

b) The virus exists intracellularly as a nucleoprotein complex in both the cytoplasm and the nucleus.

c) The levels of linear DNA, which are the precursors to integration, are affected only to a limited extent, if at all, in restrictive infections. Circular viral DNA, which does not play a role in integration, is greatly reduced.

d) Virus production by cells transfected or microinjected with proviral DNA is not affected by \( Fv-1 \). In other words, in order for \( Fv-1 \) to have an influence, the viral DNA must be associated with other viral components which are missing in "naked" DNA. This component is the \( Fv-1 \) determinant which precisely mapped to two adjacent amino acids on the p30 protein which codes for the major capsid protein.

Taking these facts into account, we can postulate two models. In the first, we assume that at some point after entry of the virus, the \( Fv-1 \) gene product interacts with the capsid structure, perhaps only weakly. This reflects the "relative resistance" phenomenon and the fact that the gene product appears to be lost when the nucleoprotein integration complexes are purified. The interaction might effectively lock the viral DNA inside the complex by
altering its conformation, so when the complex reaches the host DNA, the viral DNA may not be able to escape and integrate. Alternatively, the complex may just be physically held away from the host DNA by Fv-1. The mistakes, or dead-end byproducts of integration (the circles) could not be formed if the viral DNA is not released from the capsid structure. This would be reflected by the lack of circles in restrictive cells, even though the levels of linear viral DNA appear to be unaffected.

A second model is suggested by the need for mitosis to occur for integration to take place (Roe, 1993). The parallels between infection in aphidicolin-arrested cells (see section 1.2.3) and Fv-1 restricted cells are remarkable and are as follows:

1) In both systems, normal amounts of linear viral DNA are observed.
2) There is a reduction, or complete block in the case of aphidicolin, of integration of the provirus.
3) The formation of circular viral DNA is not seen.
4) Integration-competent complexes can still be recovered in each system, so no physical degradation seems to occur.

Fv-1 restriction appears to precisely mimic the physical barrier of the nuclear membrane. This would suggest that Fv-1 might act by preventing access to the nuclear compartment during the time of nuclear membrane breakdown. But mitosis must nevertheless take place. It is known that during the stage of mitosis known as prometaphase, nuclear envelope proteins become highly phosphorylated and thereby cause the disruption of the membrane. This process is not a complete destruction of the membrane, but rather the membrane is broken into fragments which remain visible around the spindle throughout mitosis. In telophase, the final stages of mitosis, the envelope
fragments reassemble around the two groups of daughter chromosomes (Alberts et al, 1983).

So, during a non-restrictive infection, the virus complex would travel to the nuclear membrane, wait for mitosis, enter the cell and then integrate. In a restrictive infection, the virus might still travel to the nuclear envelope, but by the interaction of Fv-1 and p30, the virus complex could become more strongly attached to the envelope (or other structures outside the nucleus), than would otherwise be the case. In other words, Fv-1 could act like a kind of "glue". On envelope breakdown during mitosis, the complex might not be able to free itself in order to integrate. Following mitosis, the nuclear envelope would then reform, with the virus, perhaps still attached to the external surface of the nucleus, unable to complete its life cycle. This again would account for the drop in integrated provirus and lack of circle formation.

The major objection to the cell cycle model is that many investigators have observed linear viral DNA inside the nucleus of restricted cells. It has been suggested that this observation could well be an artifact of the cellular fractionation process (Pryciak and Varmus, 1992). The linear DNA, apparently present in the nuclear fraction, may only be attached to the outside of the nuclear membrane, perhaps in the complex framework of the cytoskeleton or the endoplasmic reticulum which is continuous with the nuclear envelope. This would again be supported by the fact that no circles are seen in arrested or restricted cells (which would be a marker for nuclear entry) and that this is the result of the failure of the linear DNA in the capsid complex to enter the nucleus in the first place.
1.8.1 Models of \textit{Fv-1} Action in Relation to Kinetic Data

Because restriction can be overcome by a high multiplicity of infection, perhaps even by the pre-infection of just a single virus particle (Duran-Troise \textit{et al}, 1977), this would mean that a critical amount of \textit{Fv-1} gene product must be "used up" in that first encounter, thereby leaving a second virus free to carry out a successful infection. Both the models are consistent with this two-hit phenomenon observed in restrictive infections, i.e., the abrogation effect of the first virus (see earlier). The one hit curves occasionally observed in restrictive infections might possibly be due to the virus fortuitously evading the \textit{Fv-1} gene product, an event which might be seen more often if infection occurs during mitosis when the nuclear envelope is not intact. It would be interesting to synchronize the division of restrictive cells and to determine if successful infection occurs more frequently at a particular point in the cell cycle.

1.9 Approaches to Cloning \textit{Fv-1}

These above models are obviously speculation but could be tested if the \textit{Fv-1} gene were cloned, its sub-cellular location identified and the nature of its molecular interaction with the capsid resolved. Unfortunately, there is no obvious way to go about the task of cloning \textit{Fv-1}.

1.9.1 The Cellular and Biochemical Approach

At least twenty years ago the first experiments were carried out in an attempt to isolate the \textit{Fv-1} gene product. It was possible to block viral infection in infected permissive cells by fusing them with \textit{Fv-1} restrictive cells within a few hours after infection (Tennant \textit{et al}, 1974). It was suggested, therefore, that \textit{Fv-1} restriction might be a post-penetration event. Subsequently, the transfer of \textit{Fv-1} resistance was achieved by means of crude
cell lysates (Tennant et al, 1974). Up to 500 \( \mu g/ml \) of cellular protein was used to transfect susceptible cells which gave a maximum of 50-80% inhibition. This activity was lost after two hour incubation at 37°C or after 30 minutes at 56°C. The most effective time period to inhibit infection was 2 hours before or after infection with MuLV. (Yang et al, 1978) tested subcellular fractions for \( Fv-1 \) activity and found that cytoplasmic RNA could transfer the activity. The extract was effective for a five to six hour period at a time of -2 to +5 hours after infection. The active component in the cell extracts appeared to be a polyadenylated RNA with a sedimentation coefficient of 20-22 S. It was functional only if added within a narrow time period before and after infection, suggesting that restriction occurred early in the infection cycle. Genetic analysis revealed that the resistance transfer activity correlated with segregation of the appropriate \( Fv-1 \) genotype of the embryo cultures used (Tennant et al, 1976). This was confirmed using RNA preparations from pooled embryos of known genotype to transfer \( Fv-1 \) resistance. These assays involved transfection of susceptible SC-1 cells with the RNA preparations, followed by the XC plaque assay (Rowe et al, 1970). Although plaquing efficiency was poor, it was clear that RNA from Balb/c mice (\( Fv-1^{b/b} \)) specifically inhibited N-tropic virus; NIH (\( Fv-1^{n/n} \)) RNA restricted B-tropic virus and F1 hybrids of C3H (\( Fv-1^{n/n} \) x C57BL/6 (\( Fv-1^{b/b} \)) restricted both N- and B-tropic virus (Rascati et al, 1981). Further attempts to identify and characterize an RNA species has not progressed in recent years, hampered no doubt by the unstable activity in cell-free extracts.

In addition to this lack of success, the \textit{in vitro} integration assays have failed to duplicate \( Fv-1 \) restriction. Nucleoprotein complexes from isolated restrictive infections are fully integration-competent, due perhaps to the product of \( Fv-1 \) being lost during preparation (Pryciak and Varmus, 1992). According to our second model, however, these complexes which were...
isolated from nuclei of restrictive cells, would have evaded restriction, and would consequently always integrate normally in vitro. Attempts to detect inhibitory activity in non-restrictive cells have met with no success. Had this approach worked, it might have provided a means whereby to purify Fv-1.

Attempts have also been made to identify Fv-1 by differential hybridization of cDNA libraries from Fv-1 restrictive and non-restrictive mouse cell lines (Naito et al, 1992). A clone was isolated which was specific to restrictive cells, but this turned out to encode a heparin-binding molecule which failed to confer Fv-1 restriction on a susceptible cell.

Another approach being attempted by a number of groups is to clone Fv-1 directly by virtue of the physical interaction thought to occur between the Fv-1 gene product and the viral p30 protein. This method relies on a novel system to detect protein-protein interactions called the yeast two- (or di-) hybrid system (Fields and Song, 1989). The yeast protein GAL4 of Saccharomyces cerevisiae, is a transcriptional activator required for the expression of genes encoding enzymes of galactose utilization. GAL4 consists of a DNA binding domain and an activation domain. The endogenous GAL4 gene is deleted and a GAL1-lacZ fusion reporter gene is introduced into the yeast genome. The two components of GAL4 are then supplied separately, encoded on two separate plasmids. The first plasmid encodes a fusion between the GAL4 DNA-binding domain and the first protein of interest. The second plasmid encodes a fusion between the activation domain and the second protein (from a cDNA library) which could potentially bind to the first protein. If the proteins do interact, the two components of GAL4 are also physically brought together, which activates transcription from the lacZ gene. (Luban et al, 1993) used this system to
detect proteins which interact with the HIV1 gag protein. It has been reported that the same group has identified a host protein which binds to MuLV gag (Alin et al, cited in Klasse et al, 1993). However, this protein bound to gag of N- and B-tropic strains and did not map to the same chromosome as Fv-1. Several other groups are known to be attempting this or a similar approach at the present time (W. Frankel, pers. comm.).

1.9.2 A Genetic Approach to Cloning Fv-1

Because no information so far available has proved useful concerning the nature of the Fv-1 gene which would help in its isolation, we planned to clone the gene by a positional approach. This relies on a detailed knowledge of the chromosomal location of Fv-1 and the fact that the gene, being dominant, should have a readily measurable phenotypic effect.

The idea was to refine the genetic map location of marker genes linked to Fv-1 by analysing a large back-cross. Markers proximal and distal to Fv-1 would be defined and DNA clones spanning this region, in which Fv-1 must lie, would be obtained. To assay these clones for Fv-1 activity, they would be introduced stably into mouse cell lines of the appropriate Fv-1 phenotype. If say, the Fv-1^b allele were introduced into a cell lacking this allele, the cell would be rendered resistant to infection with N-tropic virus.

1.9.3 The Genomic Location of Fv-1

Over twenty years ago, an association was observed between Fv-1 phenotype and mouse coat colour (Rowe et al, 1973; Rowe and Sato, 1973). Coat colour is determined in part by the b locus, with a dominant allele B (black) and a recessive allele b (brown). This locus was known to be on linkage group VIII (chromosome 4). There was also a possibility that Fv-1 might lie close to Gpd-1, a locus for an isozyme of glucose phosphate dehydrogenase. A three-
po in t cross w as perform ed to carry out linkage analysis involving segregation of Fv-1, Gpd-1 and b. The recombination frequency between Fv-1 and Gpd-1 was <1% (1/107), between b and Gpd-1 it was 37% and between b and Fv-1 it was 36%. From this work the likely gene order was b→Fv-1→Gpd-1 or b→Gpd→Fv-1.

1.9.4 Other Linked Genes

Since the mapping of Fv-1 to the distal region of chromosome 4, several other genes have been mapped to the immediate area which could be of use in the positional cloning and mapping experiments.

Recombinant inbred (RI) strains of mice have proved invaluable mapping tools. RI strains are derived by inbreeding pairs of F2 progeny from two unrelated but highly inbred progenitor strains. Each brother-sister mating at the F2 generation is the beginning of an individual RI strain. After at least 20 generations of inbreeding by brother-sister mating, each inbred strain contains a unique combination of the progenitor alleles. Using a set of these strains enables genetic markers, which are polymorphic between the progenitors, to be mapped (Bailey, 1971). Since each RI strain is fully inbred, data obtained by different investigators are cumulative. This is a useful feature for a biological marker like Fv-1, which can be difficult to type. The C57BL/6J x C3H/HeJ and C57BL/6J x DBA/2J RI sets were typed in 1977 for Fv-1 (Taylor et al, 1977). It is possible to type these strains now for linked markers, knowing that the Fv-1 typing will not need to be repeated.

RI strains were used to map all the endogenous xenotropic murine leukemia viruses (Xmvs) which are examples of endogenous C-type proviruses. These loci were defined by hybridizing a specific radio-labelled oligonucleotide to EcoRI or PvuII-digested DNA. These sites are conserved
in the proviruses within the env coding region, so the probe was able to react with uniquely sized provirus-cell DNA junction fragments. Four such proviruses were found to be closely linked to Fv-1\textsuperscript{b} (Frankel \textit{et al}, 1989; Frankel \textit{et al}, 1990). The four proviruses Xmv-8,9,14 and 44 showed no recombination with Fv-1 in 36 recombinant inbred strains and were calculated to be less than 2.85 centimorgans (cM) from each other and Fv-1. They were, however, absent in all Fv-1\textsuperscript{n} and Fv-1\textsuperscript{nr} strains tested, in other words they were linked only to the Fv-1\textsuperscript{b} allele.

Another closely linked gene codes for atrial natriuretic factor, more commonly known as pronatrial dilatin (Pnd; Seidman \textit{et al}, 1984). Pnd is a potent vasoactive peptide synthesised in mammalian atria and is thought to play a key role in cardiovascular homeostasis. The gene was also mapped to within 4.6 cM of Fv-1 (Mullins \textit{et al}, 1987).

Another gene codes for tumour necrosis factor receptor-1 (Tnfr-1). In humans, this receptor binds tumour necrosis factors α and β. These molecules are cytokines and have quite diverse actions, which include cytotoxic effects against tumour cells, activation of neutrophils, roles in regulating lipid homeostasis, induction of secretion of interleukin-1 and as mediators of inflammation and endotoxin-induced shock. Tnfr-1 was mapped to within 2.8 cM of Pnd (Goodwin \textit{et al}, 1991). Zfec23 represents a cluster of zinc-finger genes and maps to this area of chromosome 4 (Crossley and Little, 1991).

### 1.9.5 The Choice of Cloning System

Since the precise location and size of Fv-1 is unknown (if it is indeed only one gene), it seemed necessary to obtain very large clones mapping to this area in order to maximise the chance of picking up the gene and to avoid
the potential problem of "losing" any part of Fv-1. Consequently, the use of cosmid clones was rejected since, even though they are easy to manipulate, they can carry only about 40 kb. Even the P1 phage vector, which can carry up to 100 kb, was considered to have too small a capacity. The construction of a contig spanning the region of interest, a potential distance of several megabases, would require the use of yeast artificial chromosomes (YACs).

The pYAC4 vector (Burke et al., 1987) contains the minimum sequences necessary to stably maintain cloned DNA as a linear molecule in the baker's yeast Saccharomyces cerevisiae. The size of cloned DNA usually varies from 50-1000 kb. However, the capacity of YACs is evidently far greater, as demonstrated by the construction of a 2.3 Mb YAC by the in vivo recombination of several smaller overlapping YACs (Dunnen et al., 1992). A 3 Mb YAC has also been constructed (R. Rothstein, pers. comm.).

![pYAC4 Yeast Artificial Chromosome Vector](image)

**Figure 1.3** The pYAC4 Yeast Artificial Chromosome Vector

The vector contains a bacterial origin of replication (ori) and ampicillin resistance gene (amp\(^r\)) for propagation and selection in *E. coli*. A functional centromere (CEN4) confers mitotic stability and correct meiotic segregation (by providing sites of attachment for the spindle) to the linear construct in the yeast host, at an average copy number of one per cell (Clarke and
Carbon, 1980; Murray and Szostak, 1983). An autonomous replication sequence (ARS) contains a functional origin of replication which initiates replication only once during the cell cycle (Stinchcomb et al, 1979; Struhl et al, 1979). Telomere (TEL) sequences at each end of the YAC contain the physical ends of chromosomes and allow replication and maintenance of the linear molecule (Szostak and Blackburn, 1982). Also present on the YAC are two yeast markers (TRPI and URA3) which allow the selection of YAC transformants. The cloning of DNA into a YAC involves the initial linearization of the vector with BamH1, which removes the HIS3 "stuffer" fragment. Exogenous high molecular weight DNA is then cloned into the EcoR1 site which is located in the intron of the yeast SUP4 gene. This encodes an ochre suppressing tRNA^Tyr which suppresses the red colony colour phenotype (to white) of the ade2-1 ochre mutation in the yeast host AB1380. As a result, yeast transformants containing YACs with an insert are usually red due to the disruption of the SUP4 gene. Thus, yeast can be transformed with very large segments of exogenous DNA which can be stably maintained under selection. The various methods for creating YAC libraries will not be discussed here. Several groups have undertaken this rather difficult task and have subsequently made these libraries available for screening in the last three years.

1.9.6 Introducing YACs into Mammalian Cells
Once YACs containing particular marker genes were isolated, the crucial step would be to introduce them into a suitable cell line. This would enable a viral replication assay to be carried out in order to determine whether the exogenous DNA was able to confer an altered Fv-1 phenotype; in other words, whether it carried Fv-1. Prior to this, however, the YACs had to be fitted with a selectable marker in order to identify transformed mouse cells. YAC DNA can be manipulated relatively easily in the yeast host by a
combination of homologous recombination and the use of yeast selectable markers. Using such methods, it is possible to generate alterations ranging from single base changes, deletions, gene replacement or insertions of any size (Rothstein, 1991). A most useful technique is the ability to insert a mammalian selectable marker (for instance the neomycin resistance gene, neo") into the YAC arm, or if required, into the cloned DNA itself, by homologous recombination between repetitive or other known sequences (Pavan et al, 1990a).

Once the YAC of interest has been modified to carry the mammalian selectable marker, it is ready to be introduced into the cell. In some cases, where the YAC itself contains a gene which can be selected for, such as hypoxanthine phosphoribosyltransferase (HPRT; Huxley et al, 1991) or the phosphoribosylglycinamidine formyl transferase (GART; Gnirke et al, 1991; Gnirke and Huxley, 1991) the use of additional selectable markers can be avoided.

1.9.7 Methods to Introduce YACs into Mouse Cells

There are several methods for introducing YACs into mammalian cells. One is by traditional calcium phosphate precipitation (D'Urso et al, 1990; Eliceiri et al, 1991). In the first study the YAC used was 40 kb and in the latter case, two YACs were used but their sizes were only 40 and 70 kb. Although this method proved to be quite efficient, it is unlikely that large YACs would be transferred intact by this method due to the extensive shearing of DNA. Another method is lipofection (Choi et al, 1993; Gnirke et al, 1991; Strauss and Jaenisch, 1992) which, as with the first method, requires the purification of YAC DNA. The risk of substantial shearing of large YACs is great, consequently this is not the method of choice.
Microinjection of YACs into mammalian cells has also been extensively used as shown by (Gnirke and Huxley, 1991; Gnirke et al, 1993). More recently this method has been used to generate transgenic mice (Schedl et al, 1992; Schedl et al, 1993a; Schedl et al, 1993b). Again, the disadvantage of this method is that the YAC DNA must be purified in large quantities and is sheared to a greater or lesser extent during DNA preparation and transformation as it passes through the needle. For many mammalian genes, which are less than ~50 kb in length, this approach could, however, be quite acceptable. In addition, the micro-injection facilities to carry out the large numbers of such experiments needed to achieve complete integration of many different YACs were not immediately available. With such methods, the YAC DNA must generally be isolated by gel purification. The main drawback of using gel purified YAC, however, is the low yield of DNA. This has been overcome by the recent system which can amplify YAC copy number 10-20 fold (Smith et al, 1990).

In our case, because the size and location of Fv-1 in any of the YACs relative to the marker genes was unknown, a very gentle method of YAC transfer seemed desirable in order to avoid shearing. This can be accomplished by fusion of yeast spheroplasts with mammalian cells using polyethylene glycol (PEG). This can result in stable transfer of DNA from the yeast cell (a plasmid, for example) to the mammalian cell (Ward et al, 1986).

An impressive demonstration of such a technique was the transfer of an entire yeast chromosome (from Schizosaccharomyces pombe) into mouse cells, resulting in this chromosome being able to replicate autonomously in the mouse cell (Allshire et al, 1987). This method has proved very useful using a variety of mouse cell lines and was first used for YAC transfer by (Travner et al, 1989). Several successful attempts have already been made to
transfer human-derived YAC DNA from yeast to mouse cells using YACs modified to carry the bacterial aminoglycoside phosphotransferase (neo') gene linked to mammalian control elements (Pachnis et al, 1990; Pavan et al, 1990b; Davies et al, 1992) or by transferring the larger selectable GART or HPRT gene (Gnirke et al, 1991; Huxley et al, 1991; Jakobovits et al, 1993). The evidence to date, suggests that when stable integration is achieved, it occurs at a single, unique site in the genome (Pachnis et al, 1990). Also, even with its low frequency of transformation, at a certain, if variable frequency, the entire YAC was shown to integrate. Since there is no extraction and subsequent shearing of yeast DNA during this process, there might be no theoretical upper limit to the size of YAC which may be introduced into a mammalian cell. In addition, this technique does not require any specialized equipment.

A potential problem in using total yeast DNA is that upon introduction into mammalian cells, much of the yeast genomic DNA is stably retained (Pavan et al, 1990b). The yeast DNA can be detected on a Southern blot by hybridizing with a yeast repetitive element (Ty-1) of which there are about 30 copies in the genome. For many experiments, the yeast DNA is of no consequence; it represents a tiny percentage of DNA when compared to the mammalian genome. However, even if this DNA proves to be largely inert in mammalian cells, each chromosomal segment represents a potential insertion mutagen.

Results presented here show the work done to refine the genetic map position Fv-1. The most closely linked of the marker genes were used to isolate YACs made from Fv-1b mouse DNA, several of which were then introduced into cell lines lacking this allele. Using this approach, it was hoped that Fv-1b would be discovered on one of the clones.
2.1 Plasmid Propagation and Isolation in *Escherichia coli* (*E. coli*)

Plasmid propagation and isolation were all performed using standard techniques and media (Sambrook *et al.*, 1989). Routine transformation of bacteria was performed by the CaCl₂ method. Plasmid DNA was isolated by the alkaline lysis mini-prep method.

**2.1.1 Bacterial Transformation by Electroporation**

For bacterial transformations requiring a high degree of efficiency, for example in a rescue experiment when colony numbers were expected to be low, electroporation was used. Bacteria were prepared as follows: 1 litre of L-broth was inoculated with 20 ml of a fresh overnight culture and then incubated at 37°C with shaking until the culture reached an O.D.₅₅₀ of 0.6. The culture was centrifuged at 4000 rpm for 10 minutes at 4°C, then the pellet was resuspended in an equal volume of ice-cold water and centrifuged again. The pellet was washed once more with water, then twice with 50 ml of 50% glycerol in water. The pellet was finally resuspended in 2 ml 50% glycerol and 100 µl aliquots were stored at -70°C.

For the transformation procedure, an aliquot of bacteria was thawed and 80 µl placed in an electroporation cuvette (Bio Rad or Invitrogen: 0.1 cm gap) and held on ice until required. Transforming DNA (≤5 µl) was added, the cuvette was tapped to mix the solution and placed into the electroporator (Invitrogen Electroporator). The bacteria were pulsed using the following parameters: 1500V, 150Ω, 50µF, 50mA, 50W. Immediately after pulsing, 1 ml of SOC medium was added and the bacteria were incubated at 37°C for 1 hour. An appropriate volume was spread onto L-agar plates containing...
ampicillin and methicillin (each at 50 µg/ml) or kanamycin (50 µg/ml). The plates were incubated overnight at 37°C.

2.2 Digestion and Isolation of DNA Fragments From Plasmids

Plasmid was digested in a 20 µl volume with the appropriate restriction enzymes (Boehringer or NEB) according to the manufacturer’s instructions. One of two methods was used to gel purify DNA. The first was to electroelute the DNA onto DEAE paper, from which the DNA could be recovered. This method worked best for fragments over 500 bp in size. For fragments smaller than 500 bp, the spin-column method was used. DNA size markers can be seen in Appendix 6.

2.2.1 Purification by Electroelution Onto DEAE Paper

After digestion, plasmid DNA was separated by agarose gel electrophoresis. The percentage of the gel (0.8-2%) depended on the size of DNA to be isolated. The bands were visualised under long-wave UV and an incision made with a clean scalpel blade immediately in front of the band to be isolated. A piece of DEAE-cellulose NA45 membrane of the correct size was inserted into the incision. The membrane had been previously prepared as follows: strips of membrane were soaked for 5 minutes in 10 mM EDTA pH 8.0 which was then replaced with 0.5 N NaOH for a further 5 minutes. The strips were washed six times in sterile water and finally stored in sterile water. The DNA was electrophoresed onto the membrane for 5 minutes at 100V. The membrane was removed, rinsed briefly in sterile water to remove pieces of agarose, placed in 400 µl elution buffer (1.5 M NaCl, 20 mM Tris-HCl pH 7.6, 1 mM EDTA) and incubated for 30 minutes at 65°C. The paper was discarded and the buffer extracted with phenol/chloroform. The DNA was ethanol precipitated before being resuspended in 20 µl of water. The isolated DNA was not generally quantified, however, an aliquot was run on an agarose gel to confirm its size and integrity.
2.2.2 Purification by Spin-Column

For DNA fragments of less than about 500 bp, the method of choice for purification was by the spin-column method. DNA was digested and separated by gel electrophoresis for a suitable time. The gel was visualized under long wave UV and a rectangular section (about 1 cm x 2 cm) along the length of gel was cut out with a scalpel blade directly in front of the DNA band. The space was filled with molten 1% SeaPlaque LMP agarose (~2 ml). After this had set, electrophoresis was continued until the DNA had migrated into the LMP agarose. The DNA was then cut out of the LMP agarose and placed in a 1.5 ml Millipore Ultra-Free MC micro centrifuge tube fitted with a disposable 0.45 µm filter unit. The tube was placed on dry ice until the agarose had frozen after which the tube was centrifuged at room temperature for 10 minutes. The agarose particles remained in the filter unit, the gel buffer containing the DNA passed through the membrane and collected in the bottom of the microfuge tube. The DNA was phenol/chloroform extracted, ethanol precipitated and finally resuspended in water.

2.3 Sub-Cloning into M13 and Sequencing

Bacteriophage M13 DNA (either M13mp18 or M13mp19, depending on the orientation required) was digested with the appropriate restriction enzymes, phenol/chloroform extracted, ethanol precipitated and then resuspended in 10 µl of water. The M13 DNA was not quantified before use in the ligation procedure, nor was it purified from the excised fragment of linker DNA. Ligation was then carried out using T4 DNA ligase (BRL) according to the manufacturer's instructions in a 10 µl reaction volume which contained 4 µl of the purified plasmid fragment and 4 µl of digested M13 DNA. The ligation was incubated at room temperature for 2 hours then stored on ice until
required. JM101 F' bacteria were made heat-shock competent and transformed with 1/2 or 1/20th of the ligation mix. The transformed bacteria were transferred to 3 ml molten soft agar (7 parts LB agar to 8 parts LB medium) maintained at 42°C. To this mixture, 100 μl of bacteria from the 1:200 culture (which had also been grown, with shaking for 120 minutes, at 37°C), 40 μl of 5-bromo-4-chloro-3-indoyl-β-galactosidase (X-gal; 40 mg/ml in dimethyl formamide) and 40 μl of isopropyl-β-D-galactopyranoside (IPTG; 0.25M in water) were added. The mixture was swirled gently and poured onto a plate of L agar. Once the agar had solidified, the plates were incubated overnight at 37°C.

Clear plaques (representing M13 DNA carrying an insert) and a control blue plaque (representing M13 DNA with no insert) were picked. Single-stranded DNA was prepared by the PEG precipitation method as described (Sambrook et al, 1989). The USB Sequenase kit and protocol were used to sequence the single-stranded DNA. The sequencing primer used, unless otherwise stated, was the -40 universal primer supplied with the sequencing kit. Prior to loading onto a pre-run 6% denaturing polyacrylamide gel the samples were heated to >70°C for 2 minutes. Gels were run for 3-8 hours at 65 W, fixed for 20 minutes in a solution of 10% methanol and 10% glacial acetic acid, dried and exposed overnight to X-ray film.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 Design and Preparation of primers
When new PCR primer pairs were designed, whenever possible, they were 20 bp in length, with a G or C at the 3' base. Also a GC content of around 50% was desirable, which permitted an annealing temperature of at least 58°C. Oligonucleotides were synthesised and supplied detritylated and deprotected
in 2 ml ammonia. The oligonucleotide solutions were transferred to plastic universal tubes and 4 ml of water was added to each. The tubes were placed in dry ice on a 60° slant and the contents allowed to freeze, after which they were lyophilized overnight and resuspended in 400 µl sterile distilled water. They were centrifuged in a microfuge for 30 seconds to remove debris and the supernatant was retained. Optical density of a 1/400 dilution was determined at 260 nm. The following formula, from the USB Sequenase protocol, was used to calculate concentration: pmoles/µl = OD_{260}/0.01 x number of nucleotides. All oligonucleotides were stored at -20°C.

2.4.2 Setting Up PCR Reactions
Reactions were generally set up on ice in 0.5 ml Eppendorf tubes and contained 5 µl 10x buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3 and 10 to 25 mM MgCl₂), 5 µl 10x dNTPs (2 mM each of dATP, dCTP, dGTP and dTTP), 0.5 µl of each primer (20 pmoles/µl), a known amount of DNA and 0.5 µl AmpliTaq (2 units). The reaction volume was made up to 50 µl with water and overlayed with two drops light liquid paraffin from a yellow tip. If reactions of a different volume were required, then the ingredients were adjusted proportionately. The tubes were then placed in the thermal cycler (Techne Dri-Block or Perkin-Elmer) where the reactions proceeded under the control of a selected series of programmes. Reaction conditions for individual PCR reactions are given where they are described. On completion of cycling, the PCR sample was run on an agarose gel, visualized under UV, then photographed and/or gel purified as necessary.

2.5 Screening Yeast Artificial Chromosome Libraries by PCR
Yeast artificial chromosome (YAC) libraries of mouse genomic DNA were screened by PCR to detect specific target DNA sequences through a hierarchy
of pooled samples. Libraries have been constructed in several laboratories and DNA samples for screening were sent on request. An example of the basic construction of a YAC library is as follows: after transforming yeast spheroplasts with YACs, individual clones are transferred onto growth medium in the wells of microtitre plates. If there are a total of 300 96-well plates, this would give a total number of clones of 28,800. DNA is prepared from each well, then the row and column samples are pooled from each plate to give 12 column and 8 row pools (20 samples per plate in all). An aliquot from each is taken and pooled into a single simple pool. One simple pool therefore represents a single microtitre plate. Complex pools are then generated by further pooling aliquots of the simple pools in sets of 20. Because there are a total of 300 simple pools (from 300 plates), 15 complex pools are created.

In the first stage of screening, the panel of 15 complex pools is assayed by PCR. Each positive leads into the second stage simple pools, of which there are 20. The third and final stage is to screen the array of row and column pools. A positive result at this level in both row and column would identify a clone strain coordinate containing the target DNA of the PCR reaction. However, because the YAC strains were not necessarily purified to single colony isolates prior to inclusion in the library, some of the YAC samples (about 5%) might represent more than one YAC clone. Colony PCR was used to establish clone purity (see section 2.6).
2.5.1 The Princeton YAC Library

The first library was constructed at Princeton University, Princeton, NJ from C57BL/6J (Fv-1b) female mouse genomic DNA prepared from spleen cells. The DNA had been partially digested with Eco RI and cloned in the yeast host strain AB1380 using the vector pYAC4 (Burke et al., 1991; Rossi et al., 1992). 28,000 YAC strains with an average insert size of 250-280 kb had been grown, which represented the equivalent of 2.8 haploid mouse genomes. Screening was carried out as indicated in three stages after successful completion of a series of controls. Upon sending the final results, Princeton sent culture stabs of the requested clone strains. At a later stage, an additional series of 19,200 YACs were made available as part of the Princeton library. These clones were prepared as before, however the insert size was now an average of 650 kb and provided the equivalent of approximately
four haploid mouse genomes of cloned DNA. Screening of this library was also by the pooled hierarchy PCR except the first stage consisted of 25 super pools, followed by 28 sub-pools for each first-round positive. The sub-pools were arranged as 8 microtitre tray pools, 8 row pools and 12 column pools. A PCR positive reaction in each of the sub-pool sections, enabled the identification of a single clone coordinate.

2.5.2 The St Mary's YAC library

This library was constructed in essentially the same way as the Princeton library from C57BL/10J mice (Chartier et al, 1992) and contained 41,568 clones with an average insert size of 240 kb, representing a greater than threefold coverage of the mouse genome. However, the yeast host used was a \textit{Rad52} strain. This strain is recombination deficient and so will theoretically reduce the frequency of YACs containing non-contiguous DNA.

2.5.3 Preparation of DNA from the St Mary's YAC Library.

The library was screened by PCR in a similar scheme to that of the Princeton libraries. DNA samples for all three stages of the Princeton library screen were sent through the post. However, the DNA for the final rows and columns screen of the St Mary's library had to be prepared for each simple pool positive found.

Each simple pool corresponded to two microtitre plates, i.e., a total of 192 colonies which had been grown on aAHC medium (a rich medium deficient in uracil and tryptophan, thereby selecting for the YAC arms; see Appendix 1). To each well, 200 µl aAHC was added and the yeast were resuspended by pipetting. Then 80 µl was aliquoted into the corresponding "row tube" and 80 µl into the "column tube". The microfuge tubes (28 in all;
12 "column tubes" and 16 "row tubes") were centrifuged for 5 minutes. The yeast were resuspended in water and centrifuged again. The pellet was then resuspended in 200 μl of lysis buffer (0.5 U/μl lyticase [Sigma L-5263; 50 U/μl in 50% glycerol, stored at -20°C], 0.45% Tween 20, 0.45% v/v NP-40, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.4 mM MgCl₂, 0.01% w/v gelatin). The suspension was incubated at 37°C for 1 hour and then heated to 100°C for 10 minutes. Finally the tubes were centrifuged briefly to remove most of the cell debris. A 2 μl volume of the supernatant was used per PCR reaction.

2.6 YAC Isolation, Growth and Storage

Yeast stab-cultures from PCR-positive coordinates in the YAC screens were plated onto aAHC medium. These were incubated at 30°C for 3 days until single colonies were visible. Plates could be stored at 4°C for several days without significant loss of yeast viability. Several colonies from each plate were analysed by PCR in order to identify pure colonies carrying the required YAC. Colonies could be assayed by PCR directly by simply removing a small number of cells from a colony on a plate with a yellow tip, and placing the cells directly into a PCR reaction mixture, vortexing briefly to disperse the cells, adding the mineral oil and starting the reaction. No special pre-reaction incubations were required. Once positives had been identified, the remainder of the colony was inoculated into 5 or 10 ml aAHC medium and grown for 24 hours at 30°C in an orbital shaker at 300 rpm for subsequent DNA isolation or YAC manipulation. Glycerol stocks for long-term storage were prepared by mixing 0.5 ml of the culture with 0.5 ml of 30% glycerol in SCE (1M sorbitol, 0.1M sodium citrate pH 5.8, 10 mM EDTA), freezing on dry ice and storing at -70°C. To revive a frozen stock, a small amount of culture was scraped away with a sterile needle and spread onto an aAHC plate and grown as usual.
2.7 High Molecular Weight DNA Preparation from YACs

A 2 ml volume of a 5 ml overnight culture was placed in a 2 ml micro-centrifuge tube and centrifuged at maximum speed in a micro-centrifuge for 30 seconds. The supernatant was discarded and a second 2 ml volume of culture placed in the tube which was spun again. The supernatant was discarded and the pellet resuspended in 2 ml SCE. The tube was centrifuged as before and the pellet was resuspended, by vortexing, in 2 ml SCE. The tube was centrifuged as before and the pellet resuspended in SCE to a final volume of 180 µl. A 45 µl volume of βSCE (50 µl β-mercaptoethanol per ml of SCE) was added followed by 100 units of lyticase. The cells were resuspended by vortexing and incubated for 1.5 hours in a 37°C water bath. Meanwhile a 1.5% low melting point agarose solution (Seaplaque™ FMC) in SCE was prepared and placed in a 45-48°C water bath to keep the agarose molten. The yeast spheroplasts were removed from the 37°C water bath and 200 µl of agarose solution was added. The cell solution was mixed gently by pipetting and immediately transferred to 80 µl moulds where the agarose was allowed to solidify. The blocks (4-5 per 5 ml yeast culture) were transferred to 8 ml of LDS solution (1% lithium dodecyl sulphate, 100 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0) in 15 ml tubes and incubated at 48°C for 24 hour with gentle rocking. The blocks could be stored indefinitely at room temperature in the LDS solution. Before the blocks were used in any way, they were washed at room temperature with 2 x 30 ml water or the appropriate buffer for at least 1 hour.

2.8 Pulsed Field Gel Electrophoresis (PFGE)

In order to visualise YAC DNA contained in agarose blocks, electrophoresis was carried out on a 1% pulsed field gel (Schwartz and Cantor, 1984; Carle and Olson, 1984; Chu et al, 1986; Barlow and Lehrach, 1987; Anand and Southern,
The LKB Pulsaphore apparatus was used and the running conditions were; 220V, 200 mA (max), switching time of 30 to 70 seconds (depending on the size-range resolution required), 0.75xTBE, with a run time of 20-24 hours. Commercial size standards (NEB) used were *Saccharomyces cerevisiae* chromosomal DNA in low-melt agarose and a λ-DNA ladder. The gel was stained with ethidium bromide, and viewed under UV light.

### 2.8.1 Pulsed Field Gel Hybridization

This was performed essentially as described (Stoye *et al*, 1991a). After photographing the gel, it was placed in 500 ml denaturing solution (0.5 N NaOH, 1M NaCl) for 30 minutes with gentle shaking, rinsed in water and placed in neutralizing solution (1M Tris-HCl, pH 8.0, 1.5 M NaCl) for 30 minutes with shaking. The gel was transferred onto 2 sheets of Whatman 3MM paper and covered with Saran wrap. This was placed on a gel drier and dried with vacuum, but no heat, until the gel was nearly flat (30-60 minutes). The drier was then set at 60°C for a further 15-30 minutes.

### 2.8.2 Preparation of Labelled Probe

DNA was labelled using the Mega Prime kit from Amersham according to the manufacturer’s instructions. Unincorporated [α-³²P] dCTP was separated from the labelled DNA as follows: the reaction volume was made up to 100 μl with TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and applied to a 1 ml G-50 Sephadex column equilibrated with TE and the eluate collected by centrifugation at 1000 rpm in a Mistral 1000 MSE bench-top centrifuge. The specific activity of the probe was determined by scintillation counting. Sheared salmon sperm DNA (1 μg) was added and the sample adjusted to 0.1 N HCl by the addition of 1/5 volume 0.5 N HCl. The probe was then incubated at 37°C for 30 minutes, after which an equal volume of 1M Tris-HCl, pH 7.5 was added. This acid incubation step has the effect of
fragmenting the probe, thus allowing it to enter the gel more easily during hybridization.

2.8.3 Hybridization

The dried gel was placed in a hybridization bag to which 5 ml of hybridization buffer was added (5xSSPE, 0.1% SDS). Sheared salmon sperm DNA (at a final concentration of 10 µg/ml) and acid-digested probe (final concentration of 2-4x10^6 cpm/ml) was heated at 100°C for 5 minutes, chilled on ice, then added to the bag. Hybridization took place overnight in a water bath at 55°C. The gel was then washed for 5 minutes at room temperature in 2x SSC, 0.1% SDS and then in the same buffer at 55°C for 1 hour. The gel was sealed in a bag and exposed to X-ray film at -70°C (with screens) for around 3-5 hours. Details of solutions SSPE and SSC are as described (Sambrook et al, 1989).

2.9 Separation of Double YACs and Transfer of St Mary’s YACs to a RAD 52 Yeast Strain

Occasionally, two different YACs were found in the same yeast cell. In order to work with the single YAC of interest, the two had to be separated. This was achieved by crossing with a non-YAC containing yeast strain of opposite mating type. Diploids from this mating were selected and forced to undergo meiosis. The four resulting haploid spores (contained in an ascus), each with a single YAC, were separated by tetrad dissection and could then be screened for the presence of the desired clone by PCR.

In order to introduce new selectable markers or manipulate the St Mary’s YACs by homologous recombination for any other purpose, these YACs had first to be transferred to a RAD52 host background, as opposed to the
recombination-deficient Rad52 strain which would not allow these modifications to take place. This was achieved by mating with a non-YAC containing RAD52 strain followed by sporulation and tetrad dissection as with the separation of double YACs.

2.9.1 Mating of Yeast
The YAC host strain is mating type a (MATa), so either YPH857 or CG379, both mating type α, were used in the cross (see Appendix 2 for genotypes of yeast strains). One fresh YAC colony was mixed with a fresh yeast colony of the opposite mating type on a YPD plate (see Appendix 1). This was left for 5-6 hours, after which some of the cell mixture was spread on minimal medium lacking histidine, uracil and tryptophan (-H-U-T; see Appendix 1) which enabled the selection of diploids. The plate was incubated at 30°C until single colonies could be picked.

2.9.2 Yeast Sporulation and Tetrad Dissection
A diploid colony was picked and grown overnight at 30°C with shaking in 10 ml of YPD. This was then centrifuged at 2000 rpm for 5 minutes and the pellet washed in 10 ml of water. The yeast were resuspended in 2 ml of water and 100 μl was removed and spread in a small patch onto a minimal sporulation plate (1% potassium acetate; 2% agar). This was incubated at 25°C for 3-5 days, at which time a small amount of the yeast was viewed under a microscope to check for sporulation (asci carrying four closely packed spores). When sporulation was judged to have reached a sufficiently high level (a minimum of ~1%) a loopful of yeast were placed in 50 μl of a 5% glusulase solution (NEN) and incubated at room temperature for 25 minutes. Half of the solution was then streaked onto a YPD plate and the spores from asci were separated using a Singer micro-dissection apparatus. The plate was incubated at 30°C until the spores had grown into colonies which could then be analysed to select for the desired genotype.
2.9.3 Tetrad Analysis

Determination of Mating Type by PCR: the mating types of the dissected spores was determined by PCR for future reference and to verify tetrads. This was done by colony PCR using the three mating type-specific primers (Huxley et al, 1990; see Appendix 3)

Selection of YACs in a lys2 background (requiring lysine): in those cases where yeast CG379 had been used in the mating, half of the spores were LYS2 (from CG379) and half were lys2 (from AB1380). Because subsequent manipulation of the YACs requires a lys2 background, the tetrads were replica plated both onto medium lacking lysine and onto aAHC and incubated at 30°C for 2-3 days. Those yeast which could not grow on medium lacking lysine but did grow on aAHC could therefore be identified as carrying YACs in a lys2 background. In addition, tetrads from St Mary’s YACs were replica plated onto another YPD plate and then irradiated with 50 kRads of γ-radiation from a cobalt source. The plate was then incubated at 30°C for 3 days. Those yeast which were recombination-deficient were killed and those which were RAD52 survived.

2.10 Yeast Transformation With Targeting Plasmids

YACs can be modified, for instance to carry new selectable markers or for insert end-rescue, by using plasmids designed to undergo homologous recombination in vivo, in precisely defined regions of the vector or insert (for example see pRV1 in Figure 5.1). Yeast cells were transformed with these linearized targeting plasmids by electroporation.

A fresh overnight culture of yeast cells was used to inoculate 50 ml of YPD. The culture was grown at 30°C, with shaking, until a cell density of approximately 1x10^7 cells/ml was reached, corresponding to an O.D.600 of
around 2.0, or as determined with a haemocytometer. The culture was
centrifuged at 3000 rpm for 10 minutes, then the cell pellet was resuspended
in water and centrifuged once again. The pellet was resuspended in 1 ml
water to which 50 µl of 0.5 M DTT was added. The cells were transferred to a
1.5 ml Eppendorf and incubated at room temperature for 15 minutes. The
cells were centrifuged at 6,500 rpm for 4 minutes and resuspended in 1 ml
electroporation buffer (270 mM sucrose, 10 mM Tris-pH 7.5, 1 mM MgCl₂). A
100 µl volume of cells was placed in a 0.2 cm electroporation cuvette to which
the transforming DNA was added (<1/10 volume). The cuvette was placed on
ice for 5 minutes. Electroporation was carried out using the BioRad Gene-
Pulser apparatus using the following parameters: 0.45 kV, 200Ω, 250µF. With
these parameters it was necessary to attach a Pulse Controller to the BioRad
apparatus. Immediately following electroporation, the cells were plated onto
synthetic medium lacking the appropriate nutrients to select for
transformants. This medium contained 1M sorbitol to ensure maximum cell
recovery. The plates were incubated for 4-5 days, at which point, individual
transformants could be isolated. Several colonies were picked and screened by
replica plating onto a second selective medium to detect targeted disruption
or introduction of a particular gene. In some cases PCR was used to identify
new sequences in clones which had undergone successful integration and
could therefore be used in subsequent YAC manipulations.

2.11 Isolation of YAC Insert Ends by “Bubble” PCR

“Vectorette" or “bubble” PCR (Riley et al, 1990; Arnold and Hodgson, 1991) is
a method to generate PCR products or DNA probes corresponding to the
extreme ends of YAC inserts using ligation-mediated PCR (see Figure 4.8 in
results and Appendix 4 for primer locations and nucleotide structure of
“bubbles” used).
2.11.1 Digestion of YAC and Ligation to "Bubble"

LMP agarose blocks which had been previously prepared for PFGE were used as the source of YAC DNA for bubble PCR. A block was taken from the LDS storage solution and washed for 1 hour in 2 x 30 ml water. The block was transferred to an eppendorf tube and heated at 65-70°C until molten. A 10μl volume (~0.5μg) of DNA/agarose was then digested overnight at 37°C with one of a series of restriction enzymes in a 40μl reaction volume. Upon complete digestion of the DNA, 160μl of water was added, the mixture was phenol/chloroform extracted and ethanol precipitated and the DNA pellet was resuspended in water. All the DNA was then added to a 50μl ligation reaction containing the appropriate "bubble" to be ligated to the insert DNA (see appendix 4 for bubble sequences). The ligation was carried out for 2 hours at 37°C, after which time the volume was made up to 200μl with water.

2.11.2 PCR Reactions

The first round of PCR was as follows: 25μl reactions were prepared in the usual way using 2μl of the diluted ligation mix and the appropriate primer combinations for either the left or right side (see section 4.5.1 and Appendix 4). A second round of PCR was then prepared as follows: an aliquot of the first round PCR was diluted 1:50 in water, 1μl of which was used to seed a second PCR. This reaction was set up as before, except that the second, internal primer combination was used for both left and right sides. Cycling conditions were as before. The samples were run on 1% agarose gels and PCR products greater than 200 bp in size were isolated by the spin-column method and resuspended in 20μl water.
2.11.3 Cloning and Sequencing of “Bubble” PCR Products

In order to generate high quality sequence data from the PCR products to allow further PCR primers to be designed, they were cloned into one of two commercially available vectors. The first was the TA Cloning™ System from Invitrogen and the second was the pGEM-T Vector™ kit from Promega. Both kits contained all the reagents and bacteria necessary for cloning and were used according to manufacturers instructions, except that half the recommended amount of vector was used in both cases. The cloning procedure takes advantage of the non-template dependent activity of thermostable polymerases used in PCR which adds single deoxy-adenosines to the 3'-end of duplex molecules generated. The vectors are designed with a single 3' T-overhang at the insert site which facilitates cloning of PCR products possessing the 3' A-overhang. Once the PCR products were cloned into the vector, the plasmids were introduced into the supplied competent bacteria by the standard heat shock method. Blue/white selection was used to identify the desired transformants, with white bacterial colonies being those containing the cloned PCR product.

2.11.4 Isolation of Single Stranded DNA for Sequencing

Both the cloning vectors contained an M13 origin of replication, which allowed single-stranded rescue after infection with the helper virus M13K07 (Promega). Fresh colonies were transferred to 3ml of TY medium and 5µl of M13K07 with a titre of ~10^{11} pfu/ml was immediately added. The culture was grown at 37°C with shaking for 90 minutes after which kanamycin was added to 60µg/ml, the culture was then grown for a further 14-18 hours. Isolation of single stranded DNA was carried out as normal, except that the entire 3ml of supernatant was processed. One third of the DNA was used in a sequencing reaction. Sequence information generated using the M13 -40 universal primer was used to design new PCR primer pairs which were specific for either the left or the right arm of the YAC.
2.12 Isolation of YAC Ends by Plasmid Rescue

A second method was also used to isolate the ends of YACs (Hermanson et al, 1991). This involved the insertion, by homologous recombination, of two plasmids into the right and left vector arms of the YAC. These facilitated the rescue, in bacteria, of plasmids from the extreme ends of the YAC insert (see Figure 4.14).

2.12.1 Yeast Transformation

The pLUS vector was targeted to the URA (right) arm of a YAC clone by digesting plasmid DNA with Sal I. The pICL vector was targeted to the CEN (left) arm by linearizing the plasmid with Sca I. Each plasmid was digested in a 50μl reaction volume and gel purified by electroelution onto DEAE paper. YAC-containing yeast were electroporated with either pICL or pLUS. The transformation mixture was plated onto synthetic medium containing 1M sorbitol, lacking lysine, uracil and tryptophan (sorb-L-U-T). PCR reactions were then carried out to determine which of the colonies obtained had arisen as a result of the desired integration event or by a non-specific integration of the plasmids elsewhere within the yeast genome, as detailed in section 4.6.2.

2.12.2 YAC Mini-Preps

DNA was prepared from yeast colonies which showed correct integration by PCR analysis. A colony was inoculated into 5 ml of -L-T medium and grown overnight, with shaking, at 37°C. The cells were collected by centrifugation at 3000 rpm for 5 minutes. The pellet was resuspended in 5 ml of SCE and then centrifuged once more. The pellet was resuspended in SCE to a final volume of 150μl in a 1.5 ml eppendorf tube. A 45 μl volume of βSCE (50 μl β-mercaptoethanol per ml of SCE) was added followed by 100 units of lyticase (2 μl of a 50 U/μl 50% glycerol stock, stored at -20°C). The cells were
thoroughly resuspended by vortexing and incubated for one hour at 37°C. The spheroplasted cells were centrifuged for 30 seconds, the supernatant discarded and the pellet resuspended in 350 μl of 50 mM Tris-HCl (pH 7.4), 25 mM EDTA, 1% SDS. The tube was incubated at 65°C for 15 minutes, with occasional vortexing. A 200 μl volume of 5 M potassium acetate was added and the microfuge tube was placed on ice for 30 minutes. The tube was centrifuged for 5 minutes, then the supernatant was transferred to a new microfuge tube and phenol/chloroform extracted twice. The DNA was precipitated by the addition of one volume of isopropanol. After mixing and allowing to sit at room temperature for 5 minutes, the tube was briefly centrifuged for 10 seconds. The supernatant was discarded and the DNA pellet allowed to air-dry and was then resuspended in 50 μl water.

2.12.3 Plasmid Rescue

A series of 40 μl digests were set up for the pICL and pLUS preparations using 10 μl of DNA and 2 μl of the appropriate enzyme. The reactions were incubated overnight at 37°C. The following day, the digests were made up to 100 μl with water, phenol/chloroform extracted and finally chloroform extracted. 200 μl ligation reactions were then set up using all of the digested DNA. The reactions were incubated at room temperature for 4-5 hours, after which the DNA was phenol/chloroform extracted and ethanol precipitated in the usual manner. The DNA pellets were resuspended in 10 μl of water and used to transform bacteria by the electroporation method described in section 2.1.1. Bacteria transformed with DNA from the left side (pICL) were plated onto L-agar containing ampicillin (50 μg/ml), whereas those transformed with DNA from the right side (pLUS) were plated onto L-agar containing kanamycin (50 μg/ml). Plasmid mini-preps were prepared from bacterial transformants, followed by restriction digest analysis with appropriate enzymes (see section 4.6.2) to determine the size of YAC insert
ends contained in the rescued plasmids. DNA fragments of interest were gel purified and sub-cloned into M13 for sequencing.

2.13 Mouse Cell Culture

Mouse cells were grown at 37°C (humidified 6% CO₂ atmosphere) in DMEM (Dulbecco's Modified Eagles Medium, without sodium pyruvate, high glucose), supplemented with 10% heat inactivated fetal calf serum, penicillin (50 μg/ml) and streptomycin (80 μg/ml). Upon reaching ~90-95% confluence, cells were split as follows: trypsin (2 ml) was added to each plate, rinsed and removed. A further 1 ml was added, and the plates were placed in a 37°C incubator for 4-5 minutes after which 5 ml of DMEM was added and the cells were resuspended by pipetting. If necessary the cells number was determined using a haemocytometer and then the appropriate volume added to fresh medium in new plates.

2.13.1 Freezing and Reviving Mouse Cells

Cells which were to be frozen in liquid nitrogen were harvested by trypsinization from nearly confluent 10 cm plates. The cells were washed in 5 ml complete medium and then resuspended in 5 ml 10% DMSO, 40% FCS, 50% DMEM (no additives). The cells were then aliquoted into 5 1.5 ml freezing vials and held at -70°C for 2-3 days before being placed in liquid nitrogen for long-term storage. To revive cells, a vial was removed from the liquid nitrogen and placed in a 37°C waterbath to rapidly thaw the cells. The volume was made up to 5 ml with complete medium following which the cells were collected by centrifugation at 1000 rpm in a bench-top centrifuge. The cells were then resuspended in 5 ml complete medium and plated onto 6 cm dishes and grown as usual.
2.13.2 Genomic DNA Preparation from Cultured Cells

Cells were trypsinized, washed in full medium and pelleted. They were then resuspended in 2 ml of RSB (10 mM NaCl; 10 mM Tris-HCl pH 7.5; 25 mM EDTA; 1% SDS and 125 µl of a 10 mg/ml pronase stock). The mixture was incubated for 2-3 hours at 37°C. A 200 µl volume of the now viscous DNA preparation was sheared by passing several times through a syringe needle, then extracted twice with phenol/chloroform. The DNA was precipitated by the addition of 2 volumes of ethanol, mixed and centrifuged for 1 minute. The DNA pellet was allowed to air dry and was then resuspended in 100 µl of water. DNA concentration was not estimated. When required, a 0.5 µl volume of this was used in PCR reactions.

2.13.3 Virus Infection of Cell Cultures

Cells were infected with virus either during virus stock production or for the Fv-1 assays. For each infection, a 6 cm plate of ~30% confluent cells from an over night incubation was prepared. The chosen volume of virus was made up to 1 ml with DMEM to which 1 µl of 15 mg/ml polybrene (final concentration of 15 µg/ml) was added. Medium was removed from the cells, then the mixture was added drop wise, with gently swirling. The cells were incubated for 3 days, at which point they were confluent. The supernatant containing the virus was then harvested for stocks or for assay. If required for stock production, the cells were split and allowed to grow further until virus production had reached the desired level, as measured by RT levels.

2.13.4 Reverse Transcripase Assay

During preparation of virus stocks from infected cell cultures or to perform Fv-1 assays on infected cells (see below), reverse transcriptase activity in the cell culture medium was measured (Goff et al, 1981) to estimate of the levels
of virus production as follows: an 800 μl volume of culture medium was placed in an Eppendorf and centrifuged for 30 seconds to remove any cells debris. From this, 600 μl of the supernatant was transferred into an 800 μl polycarbonate centrifuge tube which was centrifuged at 55,000 rpm for 20 minutes at 4°C in a Beckman TL-100 bench top centrifuge (VTi 100.1 rotor). The supernatant was decanted and the virus pellet was allowed to drain dry for a few minutes. A duplicate set of Eppendorf tubes was prepared containing 5 μl of the original culture medium. A 500 μl cocktail was prepared as follows: 50 mM Tris-HCl pH 7.5, 1.2 mM DTT, 0.3 mM EDTA, 5 μg/ml oligo dT-poly rA, 0.12% NP-40, 72 mM KCl, 1.8 mM MnCl₂, 20 μM dTTP and 0.12 μCi/μl [α-32P] dTTP. A 25 μl volume of this cocktail was added to all tubes which were then incubated at 37°C for 60-120 minutes. 5 μl of each reaction was spotted onto a grid on DEAE-81 paper. This was allowed to dry after which the paper was washed 3 times at room temperature for 10 minutes in 200 ml of 2 x SCC and finally in 200 ml ethanol. The paper was allowed to dry then exposed to X-ray film, with screen, for ~2 hours at -70°C. Grids could also be cut out and counted in a scintillation counter for radioactive quantitation.

2.14 Yeast-Mouse Cell Fusion

In an attempt to introduce the Fv-1b allele on a YAC into mouse cells, candidate YACs were fused with mouse cells lacking this allele. YACs were transformed with the plasmid pRV1 which introduced the neomycin resistance gene into the right arm of the YAC. Integration of a YAC rendered mouse cells resistant to the antibiotic G418 (Geneticin sulphate), thereby enabling the selection of cells which had undergone successful fusion events.
2.14.1 Preparation of Yeast Spheroplasts

A 5 ml volume of -L-T medium was inoculated with a single colony of yeast and grown overnight at 30°C with shaking. This particular medium was used to select for pRV1-transformed yeast containing the neomycin selectable marker in the right vector arm. The next day, 50 ml of selective medium was inoculated with 100 or 200 µl of the overnight culture and grown overnight as before. The following day, the cultures were counted in a haemocytometer and the one at a density nearest $2 \times 10^7$ cells/ml was used in the following steps. A total of $1 \times 10^9$ cells was transferred to a 50 ml conical polypropylene tube and centrifuged in a Mistral 1000 MSE bench-top centrifuge for 5 minutes at 600g at room temperature. The yeast pellet was resuspended in 20 ml of water and centrifuged as before. This was repeated with 20 ml 1M sorbitol and again with 20 ml βSCE. Spheroplasting was achieved with the use of 1000U of lyticase (stock solution of 10,000 U/ml in 50% glycerol/SCE). The yeast were incubated in a 30°C water bath until about 90% spheroplasting had occurred. The degree of spheroplasting was monitored every five minutes by mixing 20 µl of yeast with 80 µl of water (which lyse the spheroplasts) or SCE (which does not) and comparing a 10 µl volume of each under a microscope with x32 phase contrast. The spheroplasts lysed in water, leaving cell debris and any intact yeast behind. Spheroplasting usually took around 15 minutes, after which point the yeast were treated very gently. They were centrifuged for 5 minutes at 240g at room temperature and then resuspended in 2 ml of STC (1M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM CaCl$_2$). The volume was made up to 20 ml with STC and the yeast were centrifuged again, as above. This wash was repeated twice more, after which the yeast were finally resuspended in 2 ml STC and left at room temperature until required (for no longer than 1 hour).
2.14.2 Preparation of Mouse Cells

For each fusion experiment, cells from two 10 cm tissue culture dishes, at ~90% confluence, were harvested by trypsinization. The volume was made up to 30 ml with DMEM and the cells were centrifuged for 5 minutes at 130 g at room temperature. The cells were washed a further 3 times in 20 ml serum-free medium. The cells were finally resuspended in 10 ml of serum-free medium and counted in a haemocytometer.

2.14.3 Fusion

Fusion was based on published methods (Huxley et al, 1991; Pachnis et al, 1990). 1x10^8 yeast spheroplasts were placed in a 15 ml tube and pelleted for 5 minutes at 240g. The supernatant was removed and 2x10^6 mouse cells were carefully added so as not to dislodge the yeast pellet. The tube was then centrifuged for 5 minutes at 240g. The supernatant was removed and the combined pellet was resuspended in 0.5 ml PEG solution (50% PEG 1500, 10 mM CaCl₂, 10% DMSO) over a period of 1 minute by gentle mixing with the pipette as the PEG was added at a constant rate over that time. The cell mixture was incubated at room temperature for a further minute, after which 5 ml of serum-free medium was added slowly, with mixing, over a period of 2 minutes. The fusion was pelleted as above and resuspended in full medium. The fusion was plated in four 10 cm dishes. After 24 hours of incubation at 37°C, the supernatant was removed, along with unfused yeast and cell debris, and replaced with full medium containing G418 at a concentration of 800 µg/ml. Every three days, the selective medium was changed. After about 12-14 days, resistant colonies could be clearly seen. Colonies about 2 mm in diameter were isolated by removing the medium and, using sterile forceps, placing a small sterile metal cylinder, or cloning ring (with an inner diameter of 0.5 cm), around the colony. A tight seal against the dish was achieved by coating one edge of the cylinder with
silicone vacuum grease. The cells were removed by placing 50 µl of medium, from a yellow tip inside the ring and, being careful not to disturb the cloning ring, pipetting the medium up and down while scraping the end of the tip on the surface of the dish to dislodge the cells. The suspended cells were then transferred to a 6 cm dish containing 5 ml of DMEM and G418 at 400 µg/ml. Cells were allowed to grow to ~90% confluence after which the cells were trypsinized and transferred to 10 cm dishes and again allowed to grow to ~90% confluence in preparation for the Fv-1 assay and DNA preparation for PCR analysis.

2.15 Virus Stock Production for the Fv-1 Assays

Proviral clones (Boone et al, 1983) were supplied in ethanol and were as follows:

- pWN41 (N-tropic) cloned into the HindIII site of pBR322
- pWB5 (B-tropic) cloned into the HindIII site of pBR325

JM101 bacteria were transformed with the DNA and mini-preps were carried out on the plasmids in the usual manner. Restriction digests were performed to verify plasmid structure. Approximately 1 µg of each of the two plasmids was digested with HindIII to free the virus insert. The DNAs were then phenol/chloroform extracted, ethanol precipitated and resuspended in water. Self-ligation reactions were then performed using T4 DNA ligase (BRL) at 37°C for 2 hours. Mus dunni cells, at ~30% confluence were lipofected according to manufacturers instructions (BRL) with one of the viral clones. After 24 hours the cells were trypsinized and plated onto 10 cm dishes. The cells were passaged twice weekly and concentrated supernatant from each viral transfectant was assayed for reverse transcriptase activity to monitor levels of virus production. After four passages, each was then split onto four
10 cm dishes. Once the cells had reached confluence, the supernatant was removed and replaced with fresh medium. After a further 24 hours these supernatants were collected, filtered through a 0.45 µm filter and stored in 1 ml aliquots at -70°C.

NB-tropic Moloney virus was a gift from J. Price (NIMR). NIH-3T3 cells were infected with this virus as described in section 2.13.3, and virus stocks prepared as above.

2.16 **Fv-1 Assay by the Puromycin Resistance Method**

A novel colony assay was developed which bypassed the need to measure reverse transcriptase levels to monitor *Fv-1* activity. The following viruses were constructed by J.P. Stoye.

2.16.1 **Preparation of NB-Tropic MCF-Puro Virus Stocks**

Mini-prep DNAs were prepared from plasmids pBabe (Morgenstern and Land, 1990) and pMo-MCF-1 (Bosselman *et al*, 1983) in the usual manner. *Mus dunni* cells, at ~30% confluence, were co-lipofected with 10 µg of each plasmid. After 3 days, the cells were split 1/10 on a 10 cm plate and the following day, puromycin was added to the medium at a concentration of 4 µg/ml. Selection was maintained with medium change every 3-4 days. After 10 days, the cells were split once more. Upon reaching ~90% confluence, spent medium was removed and replaced with fresh medium. The following day the medium was collected, filtered through a 0.45 µm filter, and frozen at -70°C in 1 ml aliquots. These served as the NB-MCF-Puro virus stocks.
2.16.2 Construction and Recovery of N and B-Tropic MCF Virus

Mini-prep DNAs were prepared from the plasmids pWN41 (N-tropic), pWB5 (B-tropic) and p247W (Holland et al, 1985). The 5' halves of pWN41 and pWB5 were sub-cloned into pGEM3 using conserved Pst I and Hind III sites. The 3' half of p247W (MCF envelope) was similarly sub-cloned into pGEM3. The pGEM3 plasmids were then digested with Pst I and Hind III and the N or B tropic 5' halves were ligated to the 3' half of p247W resulting in N-MCF and B-MCF clones. Mus dunni cells were lipofected with the virus ligation mixture. The cells were passed five times and tested for reverse transcriptase activity to assess successful virus production. The cells were found to be RT positive by the second passage.

2.16.3 Production of N and B-MCF-Puro Virus

The RT positive cells producing N- or B-MCF virus were lipofected with pBabe and placed under selection with puromycin at 5 µg/ml. Selection was maintained for 14 days with medium change every 3-4 days. The supernatant was collected, filtered (0.45 µm) and used to infect fresh Mus dunni cells. Following infection the cells were placed under puromycin selection. After a further 12-14 days, puromycin-resistant colonies were picked, expanded and assayed for RT to determine which lines had the highest levels of activity. Upon reaching ~90% confluence the spent medium from RT positive cell lines was removed and replaced with fresh medium. The following day the medium was collected, filtered through a 0.45 µm filter, and frozen at -70°C in 1 ml aliquots. These served as the N or B-MCF-Puro virus stocks.

2.16.4 Cell Infections

Cells were plated at a density of 5 x10^5 (for 3T3FL cells) or 1 x10^6 (for L cells) per 10 cm plate and infected with NB-, N- or B-tropic MCF-puro virus.

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Following an overnight incubation, puromycin was added to a concentration of 2.5 μg/ml. Selection was maintained, with medium change every three days, until colonies were clearly visible without microscopy (~1 mm in diameter). To keep the cells for a permanent record, the medium was removed and the cells were fixed by adding 3 ml of a 1% crystal violet (R.A.Lamb #4255S), 20% methanol solution. This was left on the cells for 1 minute then rinsed off with distilled water. The plates were allowed to air-dry leaving the cells dyed purple whilst retaining their natural morphology.
Chapter 3
Genetic Mapping

\(Fv-1^b\) is closely linked to several genes, notably \(Pnd\) and four endogenous retroviral elements, \(Xmv-8, 9, 14,\) and 44. The plan was to obtain genomic clones containing the markers most closely linked to \(Fv-1\). This would result in the co-cloning of \(Fv-1\) providing, of course, that the physical distance between the markers and \(Fv-1\) was not too great. The first task, therefore, was to perform a genetic cross to refine the map position of \(Fv-1\) relative to these marker genes.

3.1 Sub-Cloning and Sequencing the Xmv LTRs and Flanking Regions

To allow typing of the Xmv in the cross and for screening of YAC libraries, a PCR assay was designed to allow the simultaneous detection of these elements. This was possible by identifying one PCR primer in the common 3' LTR region and four unique primers at different distances from the LTR corresponding to the flanking DNA for each provirus. Sequence information was therefore required from both the LTRs and the flanking DNA of the four proviruses.

The Xmv had previously been characterized using a specific oligonucleotide probe (Stoye and Coffin, 1988; Frankel et al, 1989). This made it possible to clone junction fragments into the pBS(+) vector, using a conserved EcoRI site in the 3' end of the provirus along to the next EcoRI site in the flanking region (see Figure 3.1). These cloning experiments had been performed prior to the start of the \(Fv-1\) project.
Figure 3.1 Restriction Maps of the Cloned Xmv Junction Fragments

Note: The arrows indicate the sites used for sub-cloning.
Attempts were made to sequence the plasmids directly using primers JS109 and Stoye2 in the LTR (which had been designed previously from a consensus LTR sequence (O’Neill et al, 1985; see Figure 3.2), but it proved difficult to obtain high quality sequence data. This was probably due to a combination of unpure template and unsuitable primer design. Since new primers were to be designed to amplify the Xmvs, it was essential to have completely reliable and high-quality sequence information.

For this reason, it was decided to sub-clone the junction fragments into M13 for single-stranded sequencing. Using the restriction maps of the cloned Xmvs (see Figure 3.1), suitable double digests were chosen for subcloning into M13 in the desired orientation. Sub-cloning of the Xmvs-8 junction fragment was not successful either with a SmaI/BamHI or a SmaI/HindIII digest. However, a ~3 kb SmaI/EcoRI fragment was cloned into M13mp18. A ~1.5 kb SmaI/SacI fragment of Xmvs-9 and a ~0.9 kb SmaI/EcoRI fragment of Xmvs-14 were also cloned into M13mp18. Attempts to sub-clone SmaI/BglII and SmaI/SacI fragments of Xmvs-44 were not successful, however, a ~1.5 kb KpnI/BglII fragment was cloned into M13mp19.

In all these cases, the M13 universal primer was used to sequence the U5 LTR region of the Xmvs. Sequencing of the flanking region was achieved using primers Stoye2 and JS109 (see Figure 3.2). Even though there were mismatches between these primers and the Xmvs LTRs, the single stranded sequence was clear. A new PCR primer (SB1) was designed within the U5 region which would, in conjunction with primers in the flanking region, allow the unique amplification of each proviral junction fragment.
Figure 3.2 Xmv LTRs and Flanking Sequences  (sequencing and PCR primers are indicated)
However, before new PCR primers were synthesized for each of the flanking regions, the sequence information was compared to the EMBL sequence database using the GCG FASTA computer program, to search for homology to any known sequences, especially repeats of any kind which might interfere with unique amplification of the Xmvs. No such homologies were found, so primers specific for each Xm were designed. Figure 3.2 shows the Xm sequences together with the location of sequencing and PCR primers in each case. The mismatch in the \textit{Xmv-8} LTR sequence four base pairs from the 3' end of primer SB 1 had no adverse effect on the amplification of this junction fragment. Primers and amplification conditions are listed in Appendix 3.

Primers SB 8-SB 11 were used in conjunction with SB 2-SB 5 to amplify the flanking DNA of each of the four proviruses, independently of the LTR sequences. The assays were used to enable the detection of these integration loci in genomic clones from animals which did not possess the proviruses (see section 4.2).

To conserve DNA, conditions were tested in which all four Xm sequences were amplified simultaneously. This was successful, although the band corresponding to \textit{Xmv-44} was slightly fainter than when it was amplified independently. The difference, however, was not great and all the bands were clearly visible. This PCR reaction was performed on DNA samples from various mouse strains to confirm the presence or absence of these elements and their association with the \textit{Fv-1}\textsuperscript{b} allele (see Figure 3.3).
Lane 1: øx 174-Hae III           Lane 6: DBA/2J (Fv-1n)
Lane 2: A/J (Fv-1b)             Lane 7: C3H/HeJ (Fv-1n)
Lane 3: AKR/J (Fv-1n)           Lane 8: C57L/J (Fv-1n)
Lane 4: C57BL/6J (Fv-1b)        Lane 9: 129 (Fv-1nr)
Lane 5: Balb/c (Fv-1b)          Lane 10: øx 174-Hae III

The lower lanes are all control Pnd PCR amplifications to ensure DNA samples can be amplified.

**Figure 3.3**  Xmv PCR on Several Mouse Strains

### 3.2 Genetic Mapping Studies

A large backcross was analysed to refine the genetic map of the distal end of chromosome 4 using a number of markers surrounding the *Fv-1* locus and including *Fv-1* itself. The cross was set up by J.P. Stoye using C3H/HeJ (Fv-1n) and C57BL/6J (Fv-1b) mice. Backcross mice were derived by crossing C3H/HeJ female mice with (C3H/HeJ x C57BL/6J) F1 males. A total of 368 animals were analysed. The cross was typed mainly by J.P. Stoye. My role was to analyse the Xmv loci.
3.2.1 Analysis of the Backcross

The following anchor markers were typed in all animals:

1) *Fv-1*: performed on tail fibroblast cultures.
4) *Xmv*.
5) *Pnd* (Wiseman *et al*, 1994)

*Pnd* typing was achieved by the use of primers located in the 5' untranslated region of the gene (see Appendix 3 for primers and PCR conditions) containing a polymorphic *Pvu*II site (Mullins *et al*, 1987). The amplified 257 bp product from C57BL/6J DNA (*Fv*-1) can be cleaved to give 127 and 128 bp fragments. The C3H/HeJ allele cannot be cleaved by *Pvu* II.

Prior to this cross, no recombinations had been observed between the four *Xmv*, *Pnd* and *Fv-1* (Frankel *et al*, 1989). However, the result of typing the anchor loci revealed that *Xmv*-8,14 and 44 were separated from *Xmv*-9, *Pnd* and *Fv-1* by a single recombination event.

Individuals which showed recombination between *Lck* and *Pnd* were tested with *D4Mit13* (Dietrich *et al*, 1992) and those recombining between *Pnd* and *D4Smh6b* were tested for *D4Nds5* (Cornall *et al*, 1992). 14 individuals were found to be recombinant between this second set of markers. These were subsequently typed for the internal markers *Pgd, Tnfr-1*, a zinc finger gene cluster (using the Zfec23 probe; Crossley and Little, 1991), and two intracisternal A type particle genes, *Iap5rc10* and *Iap3rc11* (Stoye *et al*, 1994). A summary of the typing for the 14 animals recombinant around *Fv-1* is shown in Table 3.1.
Table 3.1  Animals Recombinant Around Fv-1

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<td>b</td>
<td>h</td>
<td>b</td>
<td>h</td>
<td>h</td>
<td>h</td>
<td>h</td>
<td>b</td>
<td>b</td>
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<td>h</td>
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<td>h</td>
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<td>b</td>
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<td>Pnd</td>
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<td>h</td>
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<td>h</td>
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<td>Fv-1</td>
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<td>b</td>
<td>b</td>
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<td>lap3rc11</td>
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<td>h</td>
<td>h</td>
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</tr>
<tr>
<td>D4Nds5</td>
<td>ND</td>
<td>b</td>
<td>b</td>
<td>h</td>
<td>b</td>
<td>h</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>h</td>
<td>h</td>
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<tr>
<td>Gpd-1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>b</td>
<td>b</td>
<td>h</td>
<td>h</td>
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<tr>
<td>D4Smh6b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>h</td>
<td>h</td>
<td>b</td>
<td>b</td>
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<td>h</td>
<td>h</td>
<td>h</td>
<td>h</td>
<td>b</td>
</tr>
</tbody>
</table>
No recombinants were observed between \textit{Fv-1}, \textit{Xmv-9}, \textit{Pnd} and \textit{lap3rc11}. Taken in combination with data from recombinant inbred lines it was concluded that four loci should lie within 0.6 cM of each other (with a 95% probability) which would correspond to approximately 1,200 kb (Stoye et al., 1994). A genetic map of the distal end of chromosome 4 generated from this data is presented in Figure 3.5

### 3.2.2 Implications of the Genetic Mapping Results

The aim of this genetic mapping study was to determine if any marker genes were in sufficiently close proximity to \textit{Fv-1} to allow a positional cloning approach of this gene to be undertaken. Results suggested that \textit{Xmv-9}, \textit{Pnd} and \textit{Fv-1} were separated by less than 1,200 kb. The potential distance (over a megabase) which would need to be covered to be certain of encompassing all three genes was, therefore, still quite large. This effectively ruled out the use of cosmids, since very large numbers would be required to span the entire distance. The distance was small enough, however, to make use of YACs, with their large insert capacity, to physically link the genes and so make positional cloning feasible.
Figure 3.4 Genetic Map of Distal Chromosome 4
Chapter 4
Screening YAC Libraries
And Contig Building

Given that $Xmv-9$, $Pnd$ and $Fv-1$ were within 1,200 kb of each other, it was decided that YACs would offer the best chance of co-cloning $Fv-1$ with one of the markers because of their large cloning capacity (up to 2 Mb). The initial aim was to screen YAC libraries for clones containing the $Xmvs$ and $Pnd$. Having obtained these clones, the next stage was to isolate YAC-ends in order to establish overlap between the YAC to assemble a contig. The results from the genetic mapping would then be used to select a sub-set of ordered YACs from that part of the contig in which $Fv-1$ was thought to lie. This would allow us to test each candidate YAC for the presence of the gene in a systematic manner.

4.1 Screening the YAC Libraries

Three YAC libraries were available for screening. The first was the ICRF library which was made from C3H ($Fv-1^m$) DNA and contained inserts with an average size of 700 kb (Larin et al, 1991). The second library, from Princeton, was made from C57BL/6J ($Fv-1^b$) DNA with an average insert size of 250 kb (Rossi et al, 1992). The third library, from St Mary's, was made from C57BL/10 ($Fv-1^b$) DNA and had an average insert size of 240 kb (Chartier et al, 1992). The first library could be screened by hybridization, whereas the second two were screened by PCR.

The decision as to which of the libraries should be screened was based partly on the need, in future experiments, to detect the presence of an Xmv-containing YAC in a transformed mouse cell lines lacking these proviruses. The Xmvs,
being linked to the \textit{Fv-1}^b allele, could only be found on the Princeton and St Mary's YACs. The use of the Xmv YACs therefore also dictated the choice of a recipient cell line for these experiments, i.e., one lacking the \textit{Fv-1}^b allele. By transfecting suitable clones into a cell line lacking the \textit{Fv-1}^b allele, it would be possible to cause a phenotypic change in the cell line characteristic of the \textit{Fv-1}^b allele making it resistant to N-tropic virus.

The Princeton YAC library was screened for the Xmvs and with primers to uniquely amplify the second exon of \textit{Pnd} (non polymorphic; see Appendix 3). These primers were designed from sequence information obtained from the EMBL database deposited there by (Seidman \textit{et al}, 1984). The St Mary's library was screened for the following markers: \textit{Xmv-9, 44 and Pnd}.

Stringent controls had to be performed as a precondition to screening the Princeton YAC library. They were designed to ensure that the target DNA could be amplified in the concentration range expected, in the presence of excess yeast genomic DNA without spurious products obscuring amplification. Since the DNA pools were limited, it was necessary to avoid the possibility of wasting precious DNA samples. The first control was to amplify 5 ng and 0.5 ng of total mouse genomic DNA. The second control required the amplification of the target DNA in the presence of known concentrations of total yeast genomic DNA to determine if any amplification could occur from the yeast which would interfere with the mouse PCR. No interfering yeast bands were observed in this experiment, although there was an slight increase of non-specific high molecular weight product as yeast DNA concentration increased. The third control was a DNA blank (no template DNA) and finally the fourth, 200 ng of yeast on its own. A sub-set of the Xmv controls can be seen in Figure 4.1. All other primer pairs that were used to screen the libraries underwent these control assays and functioned well under identical conditions.
The screening strategy for both libraries was detailed in section 2.5. Here, an example of screening for a Pnd YAC from the Princeton library will be presented (this particular YAC turned out to be central to the success of this project). The screen can be seen in Figures 4.2-4.4. Two PCR amplifications unrelated to the Fv-1 project were performed in parallel to Pnd in the first stage complex pool screen (for the Hairless and Hox 2.9 genes).
Here, only the *Pnd* positive reactions are of interest. *Pnd* positives can be seen in Complex Pools 1, 2, 4, 8 and 11 at 323 bp.
Figure 4.3  Second-Round Simple Pool Screen for Pnd From Complex Pool 11

Figure 4.4  Rows and Column Screen for Pnd From Simple Pool 1
On completion of rows and columns PCR the clone strains were sent as stab cultures by post. Colony PCRs were performed on these samples to identify single, pure YAC clones.

The Princeton library was screened for all four Xmvs and Pnd, however, at the third stage of screening the PCR reactions failed to amplify Xmv-44. The St Mary's library was screened for this locus, and also for Xmv-9 and Pnd.

4.2 YAC Size and Stability

The size of each YAC was determined. High molecular weight DNA in agarose blocks was prepared from each YAC which could then be run on a pulsed field gel. Figure 4.5 shows typical results with several of the Xmv-8 clones. These gels were stained with ethidium bromide and visualized under UV light after which they were dried and probed with pBR322 DNA. Hybridization occurred with vector arms of the YAC, but not with other yeast chromosomes.

Most of the YACs (see Table 4.1) appeared to behave normally with respect to stability. However, YAC X8 H10 for example occasionally gave rise to two separate YACs of either 370 or 290 kb, even from the same isolate. More such cases of instability were found later. All YACs were in the expected size range for the two libraries.
Figure 4.5  Pulsed Field Gel and Autoradiogram of Xmv-8 YACs

Gel probed with pBR322 DNA. PFGE conditions: 0.75 x TBE, 50 second switch time, 22 hour run time.

Lane 1: Marker DNA  
Lanes 2-5: X8 G7 (320 kb)  
Lanes 6-8: X8 G8 (320 kb)  
Lanes 9-12: X8 C9 (310 kb)  
Lanes 13-16: X8 D4 (180 kb)
Table 4.1  Sizes of YACs Isolated from the Princeton and St Mary’s

<table>
<thead>
<tr>
<th>Locus</th>
<th>YAC</th>
<th>Size</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xmv-8</td>
<td>X8 H10*</td>
<td>370/290 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>X8 G7</td>
<td>320 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>X8 G8</td>
<td>320 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>X8 C9</td>
<td>310 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>X8 C7</td>
<td>220 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>X8 F1</td>
<td>180 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>X8 D4</td>
<td>180 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>X9 J1</td>
<td>350 kb</td>
<td>St Mary’s</td>
</tr>
<tr>
<td></td>
<td>X9 I11</td>
<td>300 kb</td>
<td>St Mary’s</td>
</tr>
<tr>
<td></td>
<td>X9 B2</td>
<td>250 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>X14 B12</td>
<td>290 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>X14 F6</td>
<td>150 kb</td>
<td>Princeton</td>
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<td></td>
<td>X44 M2</td>
<td>380 kb</td>
<td>St Mary’s</td>
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<tr>
<td></td>
<td>X44 F8</td>
<td>280 kb</td>
<td>St Mary’s</td>
</tr>
<tr>
<td></td>
<td>X44 B9</td>
<td>215 kb</td>
<td>St Mary’s</td>
</tr>
<tr>
<td></td>
<td>X44 M9</td>
<td>180 kb</td>
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<td>Pnd C9**</td>
<td>360 kb</td>
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<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>Pnd D11†</td>
<td>190 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>Pnd E10</td>
<td>180 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>Pnd D5</td>
<td>160 kb</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td>Pnd H3</td>
<td>120 kb</td>
<td>Princeton</td>
</tr>
</tbody>
</table>

*=consistently shows a second smaller YAC derived from the original
**=at least 90% loss of YAC in culture
†=mildly unstable shown by faint bands on autoradiogram below the YAC

In addition to the Princeton and St Mary’s YACs, three other YACs were obtained from the ICRF library which had been isolated by screening with a zinc finger probe known as Zfec 23 (provided by P. Little). This gene cluster was known to map to the same chromosomal region as Fv-1. The sizes of the three YACs were as follows:

Zn 5 (800 kb)
Zn 37 (550 kb)
Zn 38 (400 kb)
All three YACs were slightly unstable, Zn 5 more so than the other two, as manifest by the appearance of smaller, fainter bands on autoradiographs of pulsed field gels. Because the Zfec YACs were cloned from C3H DNA, they could not contain any of the Xmvs which were linked to \( F_\nu-1^b \). These YACs were therefore not intended for positional cloning of the \( F_\nu-1^b \) allele, but would rather be of use in the building of a contig by virtue of their large insert size.

4.3 Beginning the Assembly of a Contig

As an aid to the positional cloning of \( F_\nu-1 \), attempts were made to generate a YAC contig which it was hoped would span the most closely linked markers, as defined by the genetic mapping experiments.

None of the Princeton or St Mary’s YACs appeared to contain more than one marker gene, judging from the screening results, a fact which made contig building more difficult. To confirm this, a panel of DNA samples in LMP agarose from each of the YACs was assembled and PCR reactions carried out using the Xm v and \( Pnd \) primers. A cocktail of all the primers was added to each reaction. These PCR results showed that indeed none of the marker genes appeared on the same YAC (see Figure 4.6). However, these data obviously did not exclude the possibility that overlap could exist.
At a later stage, an additional YAC library was made available by Princeton for screening by PCR. This library was generated from C57BL/6J female mouse DNA \((Fv-T^b)\) and had an average insert size of 650 kb. Because of the apparent lack of overlap of YACs from the first two libraries, it was hoped that the large-insert clones would prove more useful in starting the construction of a contig. The library was screened for \textit{Xmv-9}, \textit{Xmv-44} and \textit{Pnd}. Several positive clones were located at the second level of screening. On performing colony PCRs on the clone stabs obtained, in several cases it was found that PCR-
positive colonies could not be identified. From those which were identified, DNA in LMP agarose was prepared and run on pulse field gels. From the hybridization results, it became apparent that all the YACs, with the exception of one or two, were highly unstable. Figure 4.7 shows three of these YACs.

![Figure 4.7 Unstable Large-Insert YACs](image)

Lanes 1-2: X44 F2  Lanes 3-4: Pnd C6  Lanes 5-6: Pnd G8

In many cases, although the yeast could be maintained in culture, the autoradiographs showed no sign of a YAC. This indicated either that these YACs were less than 40 kb in size, or that upon loss of the YAC, the vector arms containing the selectable markers had managed to integrate into the host genome. Table 4.2 shows a list of the YACs which were analysed. Despite their instability, some of the YACs could still used for PCR analysis and end-rescue experiments (see later). Only one of these YACs showed overlap; X44 F1 was positive for Xmv-8.
Table 4.2  Sizes and Stability of Several Large-Insert YACs

<table>
<thead>
<tr>
<th>Locus</th>
<th>YAC</th>
<th>Size</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pnd</td>
<td>Pnd C6</td>
<td>800-900 kb</td>
<td>Highly unstable</td>
</tr>
<tr>
<td></td>
<td>Pnd G8</td>
<td>230-290 kb</td>
<td>Highly unstable</td>
</tr>
<tr>
<td>Xmv-9</td>
<td>X9 C9</td>
<td>690 kb</td>
<td>Moderately unstable</td>
</tr>
<tr>
<td></td>
<td>X9 G11</td>
<td>450-600 kb</td>
<td>Highly unstable</td>
</tr>
<tr>
<td></td>
<td>X9 C8</td>
<td>-</td>
<td>Lost completely</td>
</tr>
<tr>
<td></td>
<td>X9 F6</td>
<td>-</td>
<td>Lost completely</td>
</tr>
<tr>
<td>Xmv-44</td>
<td>X44 F2</td>
<td>700-900 kb</td>
<td>Highly unstable</td>
</tr>
<tr>
<td></td>
<td>X44 A9</td>
<td>630 kb</td>
<td>Moderately unstable</td>
</tr>
<tr>
<td></td>
<td>X44 F1</td>
<td>500 kb</td>
<td>Highly unstable</td>
</tr>
</tbody>
</table>

4.5  Contig Building Using YAC Ends

Several methods exist to establish the overlap between YACs. One involves the use of mouse repeat PCR to generate a "finger print" for each YAC. Common products would then indicate shared DNA sequences. This approach was attempted with some YACs from the Pnd locus, however, results were unsatisfactory and overlap could not be estimated (data not shown). A second method requires the isolation of YAC insert ends for use as either DNA hybridization probes against other YACs or to generate PCR assays from sequences of these ends. It was decided to generate PCR assays from the ends as it was considered that this method might yield results more reliably and rapidly than any other method. The largest Pnd and Xmv-9 YACs were used to start the overlap analysis. The Zfec YACs, because of their large size, were also used.

4.5.1  Isolation of YAC Ends by "Bubble" PCR

Several methods exist to isolate the ends of YAC inserts, adjacent to the arms of the vector. One of these is known as "vectorette" or "bubble" PCR
(Riley et al., 1990). Despite their instability, Pnd C9, Pnd C6 and X44 A9 were used in this approach, as well as Pnd G7 and Pnd D11. See Figure 4.8 for the theory behind this method. Primer locations in the YAC vector arms and the nucleotide structure of the "bubbles" used can be seen in Appendix 4.

Briefly, YAC DNA from LMP agarose blocks was digested to completion with either Hinf I or one of a series of blunt-end cutters (Rsa I, Eco RV, Hinc II or Stu I). An appropriate double-stranded linker, or "bubble", containing an internal mismatch was then ligated to the YAC DNA. If DNA was blunt-end digested, then the Rsa I "bubble" was used and if DNA was cut with Hinf I, then the Hinf I "bubble" was used. This would give rise to some molecules consisting of a section of YAC vector (either the left or the right arm) with a fragment of insert DNA which then terminated with the appropriate ligated "bubble".

A first round of PCR was performed using the ligated mixture as the template. One of the two primers in the PCR reaction (HYAC-C for the left arm or HYAC-D for the right arm) anneals to the pBR322 region of the vector and extends, using the lower strand as its template. On reaching the ligated "bubble" the strand complementary to the "universal bubble bottom" is synthesized. Only now can the other "bubble"-specific universal primer (224) anneal and extend the other way, back towards the vector sequence, through the insert. This mechanism ensures a high degree of specificity in that only "bubbles" ligated to YAC vector sequences, via the insert, can serve as a template for PCR.
Figure 4.8 Isolation of YAC Insert Ends by "Bubble" PCR
A second round of PCR was then performed using the 224 primer together with primers internal to those previously used in the pBR322 region of the vector (LS-2 for the left side and RA-2 for the right) which further increased specificity and resulted in the synthesis of a short section of insert, flanked by a section of vector on one side and a section of “bubble” on the other. The resulting PCR products were run on a 1.5% agarose gel and PCR products of a suitable size were purified by the spin-column method and sub-cloned into a T-tailed vector. Typical second round “bubble” PCR results can be seen in Figure 4.9.

![Figure 4.9](image-url)

**Figure 4.9  Second Round “Bubble” PCRs for the Left Side of YACs
Pnd G7 and Pnd D11**

Lane 1: øx174-\textit{Hae} III  
Lane 2: Pnd D11 (\textit{Rsa I}/\textit{Rsa I})  
Lane 3: Pnd D11 (\textit{Rsa I}/\textit{Eco RV})  
Lane 4: Pnd D11 (\textit{Rsa I}/\textit{Hinc II})  
Lane 5: Pnd D11 (\textit{Rsa I}/\textit{Stu I})  
Lane 6: Pnd G7 (\textit{Rsa I}/\textit{Rsa I})  
Lane 7: Pnd G7 (\textit{Rsa I}/\textit{Eco RV})  
Lane 8: Pnd G7 (\textit{Rsa I}/\textit{Hinc II})  
Lane 9: Pnd G7 (\textit{Rsa I}/\textit{Stu I})  
Lane 10: øx174-\textit{Hae} III

Note: the first enzyme indicates the “bubble” used and the second indicates the enzyme used to digest the YAC (see Table 4.3 for further details)
Table 4.3 summarizes the YAC ends which were isolated by this method and shows the "bubble" and restriction enzyme digest combinations which were used for each case.

Table 4.3  "Bubble PCR" Results

Product sizes with all combinations of "bubble" and enzyme are shown

<table>
<thead>
<tr>
<th>Insert end</th>
<th>Digest</th>
<th>Bubble</th>
<th>YAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pnd G7</td>
</tr>
<tr>
<td>Left</td>
<td>Hinf I</td>
<td>Hinf I</td>
<td>-</td>
</tr>
<tr>
<td>Left</td>
<td>EcoR V</td>
<td>Rsa I</td>
<td>387 bp*</td>
</tr>
<tr>
<td>Left</td>
<td>HincII</td>
<td>Rsa I</td>
<td>~1500 bp</td>
</tr>
<tr>
<td>Left</td>
<td>Rsa I</td>
<td>Rsa I</td>
<td>306 bp*</td>
</tr>
<tr>
<td>Left</td>
<td>Stu I</td>
<td>Rsa I</td>
<td>~1400 bp†</td>
</tr>
<tr>
<td>Right</td>
<td>EcoR V</td>
<td>Rsa I</td>
<td>-</td>
</tr>
<tr>
<td>Right</td>
<td>HincII</td>
<td>Rsa I</td>
<td>-</td>
</tr>
<tr>
<td>Right</td>
<td>Rsa I</td>
<td>Rsa I</td>
<td>~1000 bp*</td>
</tr>
<tr>
<td>Right</td>
<td>Stu I</td>
<td>Rsa I</td>
<td>-</td>
</tr>
</tbody>
</table>

* = Sub-cloned into TA vectors and sequenced
† = Could not be sub-cloned

Only products of a certain size could be amplified with the PCR conditions used, i.e., ≤ 2 kb. Some combination of digest and bubble usually gave a PCR product corresponding to either the left or right side. One limitation of this method is that the Hinf I "bubble" cannot be used to isolate sequence from the right side of the YAC because Hinf I cuts between the HYAC-D PCR priming site and the YAC cloning site.

As indicated earlier, two rounds of PCR gives clearer results since, for example, if the RA-2 primer were used without the first round of PCR, the homology of RA-2 to the endogenous yeast Sup4 gene would result in large amounts of non-specific priming.
Single-stranded DNA was generated by rescue with helper phage M13KO7 which was then sequenced in order to design new PCR primer pairs. Primers were tested on mouse genomic DNA to determine optimum cycling conditions and to confirm the predicted size of PCR product. Detailed analysis of each YAC end rescue is given below. The sequence generated and all resulting PCR primer pairs and cycling conditions can be seen in Appendix 5.

**Pnd G7:** The 306 bp and 387 bp products from the left side of Pnd G7 were sequenced. These products were of the correct structure. However, computer searches using the GCG FASTA program revealed that approximately the first half of the sequence was homologous to a segment of a mouse B1 repeat element. The remaining sequence was not sufficient to design a suitable primer pair for this end of the Pnd G7. The ~1000 bp product from the right side of Pnd G7 was also sequenced in both orientations. From the “bubble” end, computer searches revealed that the first ~300 bp contained extensive homology to a mouse LINE repeat element. The YAC vector end of the product, however, appeared to be free of any repetitive elements. PCR primer pairs were designed which would amplify a 216 bp fragment from the right side of the YAC insert.

**Pnd D11:** The 222 bp product from the left end of Pnd D11 was sequenced and found to contain elements of a mouse B1 repeat. It was therefore not possible to create a PCR assay for the left side of Pnd D11. The ~500 bp product from the right side did, however, yield suitable sequence. Primers were designed to amplify a 255 bp fragment.
Pnd C9: Both the ~290 bp left side product and the ~270 bp right side product were sequenced, which allowed primers to be designed that amplified a 135 bp and a 214 bp fragment respectively. When the 214 bp primer pair (right side) was tested on mouse genomic DNA, no amplification was observed. This pair did, however, amplify using Pnd C9 YAC DNA taken from a LMP agarose block. An EcoR1 site was discovered between the two PCR primer sites, so it was probable that this YAC was a chimeric co-ligation product (see discussion), making amplification of genomic DNA impossible. This primer pair was of no further use for contig building. The left side primers worked well.

Pnd C6: Sequence from the 353 bp left side product revealed more mouse B1 repetitive DNA. Homology was weak however, so a primer pair was designed to amplify a PCR product of 206 bp. The right side 313 bp product gave sequence which allowed primers to be designed to amplify a 228 bp fragment. On testing the left side 206 bp primer pair using mouse genomic DNA, instead of a discrete band, a smear of DNA was observed characteristic of the amplification of a repetitive element (see Figure 4.10).

X44 A9: The ~600 bp product from the left side of X44 A9 was sequenced. Once again, computer searches revealed mouse B1 repetitive element homology with much of the sequence. The 5' end did not appear to show homology, so one primer was designed here and a second was designed inside the area of B1 homology which would amplify a 254 bp fragment. The ~600 bp product from the right side was also sequenced. This showed no B1 or LINE homology, so a primer pair was designed to amplify a 255 bp product. Figure 4.10 shows test assays using the new PCR primers from the ends of Pnd C6 and X44 A9 on mouse genomic DNA.
4.5.2 Testing YAC Chimerism

It was possible that some of the YACs analysed were chimeric, i.e., contained non-contiguous DNA. If this were the case, then the PCR products designed from the ends of these YACs would not correspond to loci on chromosome 4 and would thus interfere with contig assembly. To resolve this matter, DNA from a series of hamster-mouse fusion hybrid cell lines was made available. Each cell line contained a unique complement of mouse chromosomes so the PCR pattern generated across this panel made it possible to determine with a reasonably degree of certainty whether a PCR reaction was amplifying DNA from mouse chromosome 4. These DNA samples were a gift from Y. Boyd (MRC Radiobiology Unit, Didcot).
The hybrid cell panel included DNA from 15 hybrid lines and 6 control reactions. The chromosome distribution for the panel was not revealed for control purposes. Results were sent to Y. Boyd who indicated which particular amplification was derived from chromosome 4. Figure 4.11 shows a PCR results pattern consistent with a chromosome 4 product.

![Figure 4.11 PCR Analysis of the Hamster/Mouse Hybrid Cell Panel](image)

This particular assay is from an Xmv-9 YAC showing a chromosome 4 pattern.

The assay results for each primer pair are given below.

Pnd G7 right side (216 bp): It can be seen in Figure 4.12 that nearly every sample produced a PCR band. This non-specific priming in the absence of a chromosome 4 pattern can probably be attributed to the presence of the LINE repeat element found in adjacent DNA (see earlier). Unfortunately for the purposes of defining overlap, this PCR reaction was not usable. The left side "bubble" PCR had B1 repeat sequence, so Pnd G7 proved fruitless.
Pnd D11 right side (255 bp) and Pnd C9 left side (135 bp): these PCR reactions showed that both primer pairs were specific to chromosome 4.

Pnd C6 right side (228 bp) and X44 A9 left (254 bp) and X44 A9 right side (255 bp): all three pairs of primers gave patterns inconsistent with chromosome 4. The patterns seen suggested the presence of repetitive DNA or another chromosome (Figures 4.13 shows the result for Pnd C6 right).

In summary, therefore, only the right side of Pnd D11 and the left side of Pnd C9 gave useful PCR assays with which to help in the assembly of a contig. Results using these primer pairs on contig assembly can be seen in Table 4.5.
4.6 Isolation of YAC Ends by Plasmid Rescue

Although “bubble” PCR was simple and quick to perform, in most cases it did not yield sequence information usable for contig building. Therefore, a second end-rescue method developed by was tested (Hermanson et al, 1991). Linearized plasmids (pICL and pLUS) are introduced into yeast cells which then undergo homologous recombination into the YAC vector sequences. Once integrated, a plasmid composed of a section of insert flanked by a stretch of vector sequence can be rescued in E. coli (see Figure 4.14). The amount of DNA from which to select suitable PCR primers would, in theory, be greater than that obtained using “bubble” PCR. The advantages and disadvantages of both methods will be discussed.
Figure 4.14 Rescue of Insert DNA From the Left and Right Side of YACs Using pICL and pPLUS
4.6.1 Transfer of St Mary’s YACs into a RAD52 Background

Two of the YACs whose ends were to be rescued because of their relatively large size were from the St Mary’s library (X9 J1 and X9 I11). The yeast host has a rad52 genotype, which prevents homologous recombination. Because pICL and pLUS must integrate by homologous recombination, the YACs were transferred to a RAD52 wild-type background. This procedure is detailed in section 2.9. Briefly, YACs were mated with yeast strain CG379, diploids selected and sporulated followed by dissection of several tetrads from each YAC onto YPD plates. After several days growth, tetrads were replica-plated and selected for the presence of YACs and lys2. The rescue plasmids carry the LYS2 gene to enable selection for recombinants on a lys2 background. A set of replica-plated tetrads was also γ-irradiated. Yeast which were recombination-deficient (rad52) were killed and those which were RAD52 (wild-type) survived. Only those yeast which were lys2, survived irradiation and contained a YAC were used for recombination. Suitable YACs were taken from the original plate on which the tetrads had been dissected. A summary table of the tetrad analysis is presented in Table 4.4.

All the tetrads analysed in this case appeared to be genuine tetrads. Occasionally, during selection of tetrads from a glusulase-treated sporulation mixture, it is possible to pick accidentally a false tetrad in which free spores have grouped together to form what appears to be a tetrad. If, as in this case, all the tetrads showed a 2:2 segregation for desired characteristics (YAC or the Lys gene for example) it was reasonable to assume that they were genuine. For unknown reasons, not all of the spores having the correct genotype grew well. Consequently, only spores 1.c, 11.a, 15.a and 17.b could be used for recombination experiments.
Table 4.4  Tetrad Analysis of St Mary's YACs X9 II1 and X9 J1

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<td>lys</td>
<td>YAC LYS $\gamma$</td>
</tr>
</tbody>
</table>

YAC=Growth on aAHC medium  
LYS=growth on -Lys medium  
$\gamma$=growth after $\gamma$-irradiation  
lys=no growth on -Lys medium  
Only those spores having symbols YAC, lys and $\gamma$ can be used for recombination.
4.6.2 Transformation of YACs with pICL and pLUS

YACs X9 J1, X9 I11, Zn 5 and Zn 37 were transformed with either linearized pICL (for the left side) or pLUS (for the right side) plasmids. pICL was linearized at a ScaI site within the amp gene and pLUS was linearized with SalI within the YAC4 sequence (see Figure 4.14). YAC transformants were selected on medium lacking lysine. Colony PCR was performed on several transformants in each case to determine which of the colonies obtained had arisen as a result of the desired integration event or by a non-specific integration of the plasmids elsewhere within the yeast genome. The primers and amplification conditions used can be seen in Appendix 4. In the case of a pLUS transformant, an amplification product of 1855 bp was obtained only if integration had occurred into the YAC arm. No product was obtained from parental YAC DNA or from the pLUS vector. In the case of pICL transformants, following PCR, these products were digested with Pst I and the DNA run on a 1.5% gel. In cases where pICL integrated correctly, the PCR reaction generated an 870 bp product which could be cleaved by Pst I into 702 and 165 bp fragments. If pICL integrated into the endogenous lys2 gene, the PCR product could not be cleaved. The parental YAC did not amplify. Figures 4.15 and 4.16 show typical PCR results for X9 I11 pICL transformants and X9 I11 pLUS transformants.

DNA was prepared from the correctly transformed YACs by the mini-prep method. Individual restriction digests were then carried out using one of a series of enzymes contained in the polylinker which had been introduced with the integration of pICL or pLUS (see Figures 4.14). Each of these enzymes cut within the mouse insert DNA at varying distances from the vector arm. Following digestion, the insert-vector fragments were self-ligated and used to transform E. coli by electroporation.
Colonies 1, 2, 4, and 6 gave 870 bp PCR products which when cut by Pst I gave 705 and 165 bp fragments which was indicative of correct integration. Colony 3, however, did not cleave implying that the plasmid had integrated into the endogenous lys2 gene. Colony 5 gave no product, which suggested that this colony was a lys2→LYS2 revertant.

Only those cases in which pLUS integrated correctly was a 1855 bp product amplified.
The bacteria were plated on appropriate antibiotic-containing medium. Plasmid mini-preps were analysed from bacterial transformants, to assess the structure of the plasmids, and the length of insert DNA which had been rescued with each particular enzyme. Figure 4.17 shows an example of the digests of several X9111 plasmids rescued with both pICL and pLUS. All plasmids were digested with Eco R1 and with Eco R1 plus the enzyme used to digest the YAC DNA.

![Figure 4.17 Digests of X9111 pICL and pLUS Rescued Plasmids](image)

Lane 1: øx174-Hae III  
Lane 2: λ-HindIII  
Lane 3: BamH1 pICL rescue, digested with EcoR1  
Lane 4: BamH1 pICL rescue, digested with EcoR1 and BamH1  
Lane 5: SacI pICL rescue, digested with EcoR1  
Lane 6: SacI pICL rescue, digested with EcoR1 and SacI  
Lane 9: SacI pLUS rescue, digested with EcoR1  
Lane 10: SacI pLUS rescue, digested with EcoR1 and SacI  
Lane 11: KpnI pLUS rescue, digested with EcoR1  
Lane 12: KpnI pLUS rescue, digested with EcoR1 and KpnI  
Lane 13: øx174-Hae III and λ-HindIII
With double digests of the pICL rescued plasmids, the release of the 5.3 kb vector fragment can be observed with the remaining fragments comprising the mouse insert DNA. Similarly, a double digest of the pPLUS rescued plasmids reveals 1.8 and 2.3 kb vector fragments. The smaller fragments are the mouse insert DNA. Restriction maps of the rescued plasmids from X9 I11, X9 J1, Zn 5 and Zn 37 were constructed using the data from the single and double digests and can be seen in Figures 4.18 and 4.19.

4.6.3 Sequencing YAC Ends for PCR Primer Design

These sequences, PCR primer pairs and amplification conditions can be seen in Appendix 5.

**X9 I11:** The 2.3 kb EcoR1 fragment from X9 I11 pICL BamHI-rescued plasmid and the 1.4 kb SacI-EcoR1 fragment from the pPLUS SacI-rescued plasmid were sub-cloned into M13 and sequenced. The right side (pPLUS) revealed extensive homology to a LINE repeat element by computer analysis, thus a PCR primer pair could not be designed. PCR primers for the left side (pICL) revealed no such homology and a primer pair was designed to amplify a 244 bp fragment.

**X9 J1:** The Sac I-rescued pPLUS plasmid was sequenced directly using the pPLUS primer (see Appendix 4). The quality of the sequence was such that only the first ~200 bp could clearly be read. This, however, revealed a series of at least three 84-86 bp repeat elements each containing an Eco R1 site and several small sequence differences between the elements. A computer search showed that these tandem repeats were, in fact, zinc fingers sequences.
Figure 4.18 Restriction Digest Maps of Rescued Plasmids From X9 I11 and X9 J1
The combined information from all rescued clones is shown.

Figure 4.19  Restriction Digest Maps of Rescued Plasmids From Zn 5 and Zn 37
The mouse insert DNA appears to have been cloned on the right side at one of the repeated Eco R1 sites. The 2.5 kb Eco R1 fragment at this end of the YAC was sub-cloned into M13 and sequenced. Due to conserved Eco R1 site in the repeat, the tandem repeats were lost in this subcloning. The adjacent sequence in the 2.5 kb fragment still showed some homology to the repeats for approximately 100-120 bp. The clone was sequenced from the other end which revealed no apparent homology to either the tandem repeat or to other mouse repeat elements. However, for reasons given below, this sequence could not be used for primer design. From the pPLUS Sac I-rescued plasmid, the 5'-most 0.9 kb Sac I-Eco R1 fragment was sequenced. This showed no adverse homology against the computer data base so a primer pair was designed to amplify a 243 bp fragment.

From the X9 J1 pICL clones (left side), the 1.0 kb Eco R1-Bam H1 and the 2.5kb Eco R1 fragments were sub-cloned into M13 and sequenced from the YAC cloning end. Surprisingly, this proved to be the almost identical to the sequence just obtained from the 2.5 kb Eco R1 fragment from the pPLUS end 350 kb away, but with several point mutations in the readable sequence (see Figure 4.20). The sizes of the two 2.5 kb fragments from opposite ends of the YAC were shown to be identical within the limits of agarose gel electrophoresis. The 1.1 kb Eco R1 fragment from the Sac I pICL plasmid was sequenced in an attempt to bypass the area of repeats. This fragment, however, also showed zinc finger homology. Finally, the most 3' 1.2 kb Eco R1-Sac I fragment was sequenced. Computer searches found no homology and a primer pair was designed to amplify a 255 bp fragment.
1 gaattc...catgttctttgagttcctactatgtgcctagtagtaatg 46
1 gaattctttttctttttgagttcctactatgtgcctagtagtaatg 50
47 ctctgagatataccagagactcctgaaatgagggaaattctagttaaatca 96
51 ctctgagatataccagagactcctgaaatgagggaaattctagttaaatca 100
97 taacctttttcattgtgttttccctttacattttcctgctttctttggaaaag 146
101 taacctttttcattgtgttttccctttacattttcctgctttctttggaaaag 150
147 taaccactgctatattctctgaggtactcctatctctttatctagttcc 196
151 taaccactgctatattctctgaggtactcctatctctttatctagttcc 200
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201 agtgaacgttaaaaaagttccgaagaaaaatattaaatatccagaaacagaa 250
247 ttatatctttactataaatagttgcatttat.aaaaatctttactatttg 295
251 ttatatctttactataaatagttgcatttat.aaaaatctttactatttg 300
296 cattaatatgtaccccttttttatgatgttaattatacaagg 337
301 cattaatatgtac.......................... 314

Figure 4.20 A Comparison of Sequences Obtained from X9 J1

These sequences are from 2.5 kb fragments from opposite ends of the YAC:

Zn 5: A 1.1 kb Eco RI-Sph I and a 1.2 kb EcoR1-EcoR1 fragment from the Sph I and BamHI-rescued pICL plasmid respectively were sub-cloned and sequenced. These contained no repeats so PCR primers were designed to amplify a 1000 bp fragment. From the pPLUS Xba I-rescued plasmid a 0.3 kb Eco R1 fragment was sub-cloned and sequenced. Again this showed no adverse homologies so a PCR primer pair was designed to amplify a 229 bp fragment.
Zn 37: A 0.7 kb EcoR1-SphI fragment from the SphI-rescued pICL plasmid was sequenced. No adverse repeat element homologies were found by computer searches. PCR primer pairs were designed to amplify a 226 bp fragment. The 0.45 kb Eco R1 fragment from the Bam H1-rescued pLUS plasmid was sub-cloned and sequenced. Again, no homologies were found and PCR primer pairs were designed to amplify a 240 bp fragment.

4.6.4 Testing YAC Chimerism

As with the YAC ends isolated by "bubble" PCR, the ends isolated by plasmid rescue were tested for chimerism using the hamster-mouse hybrid panel. The PCR primer pairs were first tested on genomic DNA (data not shown) to demonstrate that they functioned correctly. PCR primers and assay conditions can be seen in Appendix 5.

The PCR results for X9 111 (left side) are shown in Figure 4.21. Unfortunately, as with several previous cases, the product appeared to be either a repeat element, which was not indicated by computer homology searching, or this end was in fact chimeric with the mouse X chromosome. This chromosome was known to appear in all samples except the negative and hamster control DNAs. These data, together with the right side of X9 111 being homologous to a LINE repeat element, meant that this YAC could not be used in contig building. Both ends of X9 J1 gave PCR patterns characteristic of chromosome 4. This was also the case for both ends of Zn 5 and Zn 37 although the levels of background in the Zn 5 reactions were quite high (not shown).
4.7 Contig Building By PCR Analysis

From nine YACs, PCR assays were only available from 8 of the possible total of 18 ends:

- Pnd D11 (right): 255 bp
- Pnd C9 (left): 135 bp
- X9 J1 (left): 255 bp
- X9 J1 (right): 243 bp
- Zn 5 (left): 1000 bp
- Zn 5 (left): 229 bp
- Zn 37 (left): 225 bp
- Zn 37 (right): 240 bp

Reactions were carried out using the above PCR reactions to determine overlaps with the same YAC panel as that described in Figure 4.6. In addition, the most stable of the new large-insert YACs were included in the PCR assays. Although the Zfec YACs had been included in the earlier PCRs, they were known to be negative for the Xmvs, since they are absent from C3H DNA (the source of DNA for the ICRF library). However, the loci at which the
proviruses integrated should be present in all strains of mice. By designing new PCR oligonucleotides from flanking sequence immediately following the LTRs from each of the Xmvs (see Figure 3.2), it was possible to use the Zfec YACs in contig building by virtue of the presence or absence of these loci in a given YAC.

Unfortunately it was evident from the X9 J1 and Zfec PCRs that repetitive elements were being amplified by these primers in almost all YACs tested. This made their use in contig building extremely limited. In several cases, the PCR products were of different sizes to those predicted from the sequence information used to design the primer pairs. This was particularly noticeable with both ends of X9 J1. Figure 4.22 shows the assay for the right side of X9 J1.

As indicated from earlier results, no two of the provirus elements appeared in the same YAC, however, on the inclusion of X44 F1 (one of the new large YACs) it was revealed that both Xmv-44 and Xmv-8 were present on this YAC. Although this YAC was highly unstable, its maximum observed size of 500 kb. This meant that the two Xmvs must lie within this distance of each other, unless of course, this YAC was formed as a result of deletions from a larger YAC. Some of the most informative reactions were those using the Xmv flanking PCRs on the Zfec YACs. These reactions revealed a precisely defined overlap between the three YACs. They did not, however, appear to contain the Pnd locus.
Figure 4.22  PCR Analysis of YAC DNAs for Contig Building

This PCR assay from the right side of X9 J1 generates a 243 bp product. This assay, however, gives products of several sizes, notably in Pnd G7 (lane 17).

1. øx174  7. X8 D4  13. X44 B9  19. Pnd D5  25. -
5. X8 C7   11. X44 M2 17. Pnd G7  23. X14 F6  29. øx174
6. X8 F1   12. X44 M9 18. Pnd E10 24. -

The results of the PCR analysis using the Xmv primers and those from the YAC ends listed above, can be seen in Table 4.5.
### Table 4.5 PCR Results Used for Contig Assembly

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</table>

**Note:** The Xmv PCRs carried out on the Zfec YACs are, in fact, reactions of the Xmv flanking PCR and detect only the locus at which the proviruses integrated.
Attempts to combine all this information into a viable contig proved to be far from satisfactory. However, a "best guess" is presented in Figure 4.23 which also takes into account information from the genetic mapping data, which separates \textit{Xmv-8, 14 and 44} from \textit{Xmv-9} and \textit{Pnd}. Zn 5 is the only YAC which spans the recombination point for certain, because of the presence of all four Xmv integration loci. We have placed \textit{Xmv-14} at the left of the contig, but this locus could also be to the right of \textit{Xmv-8} and 44. The position of \textit{X44 F1} is determined solely by the fact that it contains \textit{Xmv-8} and 44; its overlap with \textit{X9 J1} cannot be established on the basis of this data. The position of \textit{X9 J1} was not clear, because of the polymorphic nature of the PCR reactions generated from its ends. The most useful \textit{X9 J1} PCR result came from the fact that \textit{X9 J1} (right side) appeared to overlap with two of the \textit{Pnd} YACs, which was not observed with any other YAC end. The size of the product amplified from the \textit{Pnd C9} YAC (243 bp) was of the size expected, whereas that from \textit{Pnd G7} was different (~400 bp; see Figure 4.22). We therefore positioned \textit{X9 J1} so as to overlap with \textit{Pnd C9} but not \textit{Pnd G7}. There was also some contradiction in the positioning of the \textit{Pnd} YACs: in order for all these YACs to contain the \textit{Pnd} locus, the right side assay for \textit{Pnd D11} had to coincide with both \textit{Pnd G7} and \textit{Pnd C9}, even though \textit{Pnd C9} was negative for this PCR.

It had been hoped that assembling a contig would enable the systematic screening of a group of overlapping YACs, so that the area in which \textit{Fv-1} was thought to lie could be covered in the most efficient manner. Although we achieved limited success in contig building, the mapping information, which positioned \textit{Xmv-9}, \textit{Pnd} and \textit{Fv-1} within 1,200 kb of each other, proved most valuable in narrowing down the position of \textit{Fv-1}.
Figure 4.23 "Best Guess" YAC Contig

Note: The YACs are drawn to scale but the degree of overlap may not be correct. Pnd C9 and X44 F1 are included, despite their instability.
Chapter 5
Introducing YACs Into Mouse Cell Lines
and Using a Modified Assay
to Detect Fv-1 Activity

In order to assay particular YACs (derived from Fv-1$^b$ DNA) to determine if they carried the Fv-1 gene, they had to be transfected into a cell line lacking this allele. If Fv-1 was successfully introduced it would cause a phenotypic change in the cell line, i.e., causing it to restrict N-tropic virus. It was important to be able to introduce the YACs in their entirety (spheroplast fusion would achieve this) to ensure that a YAC could not be mis-characterized as not carrying Fv-1, merely because it had failed to integrate completely. The choice of cell lines used for fusion depended not only upon their ability to fully integrate YACs at a high frequency, but also for these events to be detectable simply by PCR analysis.

This chapter describes the way in which YACs were modified to allow selection following fusion, and the PCR analysis which was used to identify successful fusion events. The use of three different cell lines, the degree of success with each, and the Fv-1 assays carried out following fusion will be described.

5.1 Retrofitting YACs
The plasmid pRV1 was used to transform the YACs, which were thereby modified to carry the neo$^\ast$ gene, by the process of one-step gene disruption. This would enable their selection after entry into a mouse cell. The plasmid was digested with HindIII to remove a small stuffer section, leaving a linear fragment with truncated URA ends (see Figure 5.1).
YACs were electroporated with linearized pRV1 and plated onto -L-T medium upon which transformants grew. Usually between 50-200 colonies appeared per 500 ng plasmid. Several -L-T colonies were replica plated onto -U-T medium to determine whether the URA3 gene had been disrupted by the insertion of pRV1 (only untransformed YACs can grow only on -U-T plates). In most cases, about 70% of the picked colonies could not grow on -U-T, indicating that these transformants were appropriately targeted. This figure was in good agreement with previous experiments (Srivastava and Schlessinger, 1991). It is probable that the 30% unsuccessful transformants (ones which grew on both -L-T and -U-T) had arisen due to insertion of pRV1 into the inactive URA3 gene in the yeast host chromosome, instead of disrupting the YAC URA3 gene.
5.2 The Choice of Mouse Cell Line for the First Fusion Experiments

The YACs which were available for cloning Fv-1 were from Fv-1\textsuperscript{b/b} mouse DNA. To detect phenotypic change upon introduction of a YAC carrying this allele, the cell line to be used had to be either Fv-1\textsuperscript{null} or Fv-1\textsuperscript{n/n}. The cell line 3T3FL (Fv-1\textsuperscript{null}) appeared to be appropriate for this purpose, and could potentially also be used to clone the Fv-1\textsuperscript{n} allele from the large C3H YACs. If necessary, NIH-3T3 cells (Fv-1\textsuperscript{n/n}) were also available for fusion.

PCR reactions were carried out on 3T3FL DNA to ensure that this line was of a suitable genotype to enable the detection of YACs carrying the Xmvs associated with Fv-1\textsuperscript{b}. Figure 5.2 shows a PCR analysis of the 3T3FL DNA using both the Xmvs and Xmvs flanking PCR assays. These data revealed that 3T3FL DNA genotypically resembled Fv-1\textsuperscript{n} DNA and was therefore suitable for use in the fusion experiments. Since the mouse cell line already had the Pnd locus, a method was needed which would make it possible to distinguish the endogenous Pnd from the incoming Pnd locus on a YAC. The use of the polymorphic Pnd PCR assay used in the genetic mapping experiments made this possible. 3T3FL DNA also resembled Fv-1\textsuperscript{n} DNA with respect to its Pnd allele (data not shown). These genetic data cannot explain the fact that the original cell line from which 3T3FL cells were derived was designated as Fv-1\textsuperscript{b} (Todaro, 1972; see section 1.4.3).

5.3 Fusions Using 3T3FL Cells

The genetic mapping data indicated that of the markers used to screen the YAC libraries, Xmvs-9 and Pnd were the closest to Fv-1 (lying at most 1,200 kb from one another). At the time the fusion experiments were started however, these data were not yet available.
Consequently, the following YACs were the first to be retrofitted with pRV1: X8 G7, X14 B12 and Pnd D11. Each YAC was then fused with 3T3FL cells. For 1-2 days after the fusion, under G418 selection, the mouse cells continued to divide normally. After this time, there was a sudden halt in growth at about ~60-70% confluence. Over the next 10-12 days, the cells died and detached from the plate leaving no cell colonies.

The fusions were repeated using varying degrees of spheroplasting of the yeast cells and different PEG incubation times, these were, however,
unsuccessful. After a final experiment in which the original conditions were again tried, the Pnd D11 fusion yielded G418-resistant colonies. Of the $1 \times 10^6$ mouse cells used in the fusion, only two actively growing colonies were isolated on day 14. They were recovered with a cloning ring and expanded to $\sim 90\%$ confluence in 6 cm dishes. G418 selection was maintained throughout. The growth characteristics of these cells appeared to be normal. Several slower-growing colonies were also isolated. These, however, did not grow when expanded to 6 cm dishes, even though the cells initially adhered to the plate; these cells were discarded. The cells from the healthy colonies were split further, ready for the Fv-1 assay. It was noticed that after 3 days growth, on all but two of the plates, the growth characteristics of the G418-resistant lines appeared to have altered slightly. The cells grew rapidly, but on reaching about 60% confluence, in one or two places the cells would clump together on the plate and form small irregular-shaped foci, as though contact inhibition had been lost due to a spontaneous transformation process (Todaro, 1972). This effect did not appear to cause any problems and was not noticeable once the cells were confluent.

5.3.1 PCR Analysis of G418 Resistant Colonies

Pnd D11-transformed cells were harvested from a 5 cm dish from which DNA was prepared for analysis by PCR to determine if the fusion had been successful. To detect unique YAC sequences, several PCR reactions were carried out. Firstly for the neo$^r$ gene in the right arm, whose PCR primers gave a product size of 130 bp. Secondly, left arm-specific primers which gave a product size of 200 bp which were designed to amplify the junction between pBR322 sequence and the yeast SUP4 gene sequence in the left vector arm. Finally a PCR for the marker gene (in this case the polymorphic Pnd PCR assay) was performed. Primer sequences and amplification conditions can be seen in Appendix 3.
In both Pnd D11 lines the neo$^r$ sequence was present, as expected by their G418 resistance. The polymorphic Pnd PCR, after digestion with PvuII, showed that the original 3T3FL cell line did not have the PvuII site, leaving the full length 257 bp product. With the fusion cell line, however, in addition to the 257 bp band, the smaller band could be seen (127 and 128 bp) which was characteristic of Fv-1$^b$ DNA and resulted from the YAC PCR product having been cut at the PvuII site. The PCR for the left arm was, however, negative in both. Figure 5.3 shows the assay results for the first of these cell lines.

**Figure 5.3  PCR Analysis of the Fusion Cell Line Pnd D11**

Lane 1: øx174-Hae III  
Lane 2: Pnd PCR on 3T3FL DNA  
Lane 3: Pnd PCR on Pnd D11 DNA  
Lane 4: neo$^r$ PCR on 3T3FL DNA  
Lane 5: neo$^r$ PCR on Pnd D11 DNA  
Lane 6: Left arm PCR on 3T3FL DNA  
Lane 7: Left arm PCR on Pnd D11 DNA  
Lane 8: Positive control for left arm  
Lane 9: Xmv PCR  
Lane 10: øx174-Hae III
It was evident, therefore, that part of the YAC had not integrated, although how much to the left of the \textit{Pnd} locus was lost could not be determined. A negative control Xmv PCR was also performed.

5.3.2 Virus Infectivity Assay

The Pnd D11 resistant cell lines were infected separately with NB-, N- and B-tropic MuLV, followed by a reverse transcriptase assay to quantify the level of viral replication which had taken place in the cells. All three virus types should replicate well in the 3T3FL cells, however, if the \textit{Fv-1}^b gene is introduced on the YAC, the N-tropic virus titre should drop ~100 fold. Both the NB- and B-tropic titres acted as positive controls. Scintillation counting results of the first assay is shown in Table 5.1. The second Pnd D11 line gave similar results. The RT levels suggest that there was no reduction in N-tropic viral replication, therefore implying that the \textit{Fv-1}^b gene was not present on the section of the Pnd D11 YAC which had integrated.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Quantity of Virus Used for Infection</th>
<th>CPM from Concentrated Virus Recovered from Culture Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB</td>
<td>500 µl</td>
<td>12,808</td>
</tr>
<tr>
<td>NB</td>
<td>25 µl</td>
<td>6,611</td>
</tr>
<tr>
<td>N</td>
<td>500 µl</td>
<td>17,247</td>
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<tr>
<td>N</td>
<td>25 µl</td>
<td>5,229</td>
</tr>
<tr>
<td>B</td>
<td>500 µl</td>
<td>12,203</td>
</tr>
<tr>
<td>B</td>
<td>25 µl</td>
<td>4,957</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>5,419</td>
</tr>
</tbody>
</table>

Table 5.1 Reverse Transcriptase Assay on the Pnd D11 Fusion Cell Line (two days post-infection)
5.4 Fusions Using NIH-3T3 Cells

Due to the extremely poor levels of successful fusion using 3T3FL cells, the NIH-3T3 cell line was used as an alternative. The YACs Pnd G7 and X9 B2 were chosen for this experiment.

5.4.1 Retrofitting Pnd G7 and X9 B2

Both Pnd G7 and X9 B2 were transformed with pRV1. However, when the X9 B2 transformation mixture was plated onto -L-T, to select for pRV1 transformants, there was a lawn growth instead of discrete colonies. Upon analysis of the original X9 B2 clone stocks, it was found that the one selected for transformation was a Lys $2^->LYS$ revertant. This allowed the culture to grow in the absence of lysine. An alternative culture stock was used which worked as expected.

A problem was also encountered upon replica plating transformed -L-T colonies from Pnd G7 onto -U-T medium, to select for genuine transformants. All colonies grew instead of the $\sim 30\%$ expected. No explanation of this could be put forward, so high molecular weight DNA was prepared from several of these to see if it was possible to determine the fate of the YAC. Pulsed field gels were run and probed with pBR322. From both the ethidium stained gels and autoradiograms, it became evident that the problem was caused by the presence of two YACs of very similar size in the same yeast cell. Upon close examination of the original autoradiograms used for sizing Pnd G7, this YAC appeared to be "fatter" than other YACs, a fact which had not been noticed before. Both of them appeared to be maintained in a stable fashion. Only one of the two YACs appears to have integrated pRV1 (indicated by an increase in size; data not shown), leaving the other URA sequence in the second YAC intact, so allowing the yeast to grow on -U-T plates. Others have reported finding double YACs after retrofitting with other plasmids (Davies et al, 1992).
5.4.2 Separation of Double YACs

Because Pnd G7 was potentially very useful, it was decided to separate and isolate the YAC of interest from the culture. Pnd G7 was mated with the non-YAC containing strain YPH857, sporulated, dissected (in the same manner as that used in manipulation of the St Mary's YACs) and YACs selected on -U-T medium. Of the 20 tetrads dissected, in only five of these did all four spores grow. The reason for this poor spore viability is not known. The fact that in five cases all spores did grow, confirmed that there were indeed two YACs present, since a single YAC (because of a 2:2 segregation pattern) would have only allowed two spores to grow.

A Pnd colony PCR reaction was carried out on three tetrads to demonstrate that Pnd G7 was present in only two of the four spores in each tetrad (2:2 segregation). The other two spores gave negative results, which confirmed the presence of an unidentified YAC. A mating type PCR was also performed to confirm that the tetrads were genuine. The a and α mating type genes also segregated 2:2 as expected. Figure 5.4 shows these assays on the tetrads. It was then possible to select a pure Pnd G7 YAC which could be retrofitted.

5.4.3 Fusing Pnd G7 and X9 B2 With NIH-3T3 Cells

Fusions were carried out as before, but again, several attempts were needed before any G418-resistant colonies were obtained. Four colonies of X9 B2 cells and only one from the Pnd G7 fusion were isolated and successfully expanded. Again, several slow growing colonies were lost upon expanding onto 6 cm dishes.
Figure 5.4  Pnd and Mating-Type PCR Analysis on Pnd G7 Tetrads

Three tetrads are shown: 1st tetrad (lanes 1-4), 2nd tetrad (lanes 5-8), 3rd tetrad (lanes 9-12). The first gel shows the Pnd PCR results. The unidentified YAC did not amplify. The second gel shows the MAT PCR results. All products give a 2:2 segregation pattern.
5.4.4 PCR Analysis of G418 Resistant Colonies

PCR analysis on G418-resistant colonies was carried out as before using the neo, left side and polymorphic Pnd or Xmv-9 primers. Figure 5.5 shows the PCR assay results.

Figure 5.5 PCR Assays on NIH-3T3 Fusion Cells Lines

Product sizes: Xmv-9 (174 bp), neo (130 bp), left arm (200 bp), Pnd (323 bp)
Poly Pnd (257 bp cuts with PvuII to give 128+129 bp if the Fv-1<sup>b</sup>-linked allele has integrated; the Fv-1<sup>n</sup>-linked allele remains uncut).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PCR</th>
<th>+/-</th>
<th>Cell line</th>
<th>PCR</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1: X9 B2#1</td>
<td>Xmv-9</td>
<td>+</td>
<td>Lane 10: X9 B2#3</td>
<td>left arm</td>
<td>-</td>
</tr>
<tr>
<td>Lane 2: ..</td>
<td>neo</td>
<td>+</td>
<td>Lane 11: X9 B2#4</td>
<td>Xmv-9</td>
<td>+</td>
</tr>
<tr>
<td>Lane 3: ..</td>
<td>left arm</td>
<td>-</td>
<td>Lane 12: ..</td>
<td>neo</td>
<td>+</td>
</tr>
<tr>
<td>Lane 4: X9 B2#2</td>
<td>Xmv-9</td>
<td>-</td>
<td>Lane 13: ..</td>
<td>left arm</td>
<td>-</td>
</tr>
<tr>
<td>Lane 5: ..</td>
<td>neo</td>
<td>+</td>
<td>Lane 14: Pnd G7</td>
<td>poly Pnd</td>
<td>+</td>
</tr>
<tr>
<td>Lane 6: ..</td>
<td>left arm</td>
<td>-</td>
<td>Lane 15: ..</td>
<td>Pnd</td>
<td>+</td>
</tr>
<tr>
<td>Lane 7: Xmv-9</td>
<td>-</td>
<td>Lane 16: ..</td>
<td>neo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Lane 8: ..</td>
<td>neo</td>
<td>+</td>
<td>Lane 17: ..</td>
<td>left arm</td>
<td>-</td>
</tr>
<tr>
<td>Lane 9:øx174-Hae III</td>
<td></td>
<td></td>
<td>Lane 18: øx174-Hae III</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The PCR revealed that neo<sup>r</sup> was present in all cases, but as before, in no case did the entire YAC integrate (as shown be the absence of the left arm product). In fact, in the second and third X9 B2 colonies, neither the left side or the Xmv-9 locus had integrated. The smaller bands in the polymorphic Pnd PCR shows that the Pnd allele carried on Pnd G7 did integrate.

5.4.5 Virus Infectivity Assays
The five G418-resistant cell lines were assayed for Fv-1 activity by infection with NB-, N- and B-tropic virus as before. The results of this assay can be seen in Figure 5.6. As with the 3T3FL cells, the NB-tropic virus acted as the positive control, however in this case, the B-tropic virus acted as a negative control since NIH-3T3 cells were Fv-1<sup>n/n</sup> and therefore restrict B-tropic virus.

![Figure 5.6 RT Assays on X9 B2 and Pnd G7 Cell Lines](image)

Note: numbers indicate µl of supernatant assayed
It is evident from the autoradiogram that the levels of N-tropic virus were not affected, so the conclusion was that those sections of Pnd G7 and X9 B2 YACs which had integrated did not include a functional \( Fv-1^b \) allele. Because of the partial integration of these YACs here and in the earlier experiment, it was not possible to say that these YACs did not contain \( Fv-1^b \). This problem could only be resolved by achieving complete integration.

5.5 Fusions Using L Cells

It was vital to achieve complete integration of YACs at a reasonable frequency. 3T3FL and NIH-3T3 cells did not meet either of these criteria, so a third cell line was chosen for the fusion assay, namely L cells (LM TK\(^{-}\)). As with the previous cell lines, DNA from L cells was analysed by PCR to determine whether the introduction of \( Fv-1^b \) DNA on a YAC could be detected. The Xmrvs were shown to be absent and the polymorphic \( Pnd \) PCR was characteristic of \( Fv-1^n \) DNA (data not shown). As will be shown later, L cells were also of the correct \( Fv-1 \) genotype to be used for fusion experiments with \( Fv-1^b \)-derived YACs.

The previously retrofitted YACs Pnd G7 and X9 B2 were fused with L cells using the unmodified protocol. On the first attempt, after 10-12 days of G-418 selection, several G418-resistant colonies were observed in both fusion experiments. The growth characteristics of these cells was quite different from either of the cell lines used earlier. Colonies were composed of smaller, tightly associated cells, as opposed to the more loose aggregations of the 3T3 cells types. This also made it much easier to identify G418-resistant colonies at an earlier stage.
Eight X9 B2 and twelve Pnd G7 L cell colonies were picked and expanded. Once again, several more colonies did not survive this process. PCR assays were carried out on DNA preparations from all twenty G418-resistant lines to test for the presence of neo, the left side and the marker gene (Figure 5.7).

![PCR gel image]

**Figure 5.7**  **PCRs to Detect YAC Sequences in X9 and Pnd Fusion Cell Lines**

Lanes 1-8: X9 B2 fusion cell lines  
Lanes 9-20: Pnd G7 fusion cell lines

In 11 out of the 20 clones, both PCR products were detected. In 8 other cases, only *neo* was amplified and in one case (lane 5), neither product was seen. Since this last clone grew in the presence of G418, it is difficult to see how this line could have been negative for *neo*. This rather curious phenomenon has also been seen by other workers (C. Huxley, pers. comm.). Figure 5.8 depicts the *Xmv-9* assays. The polymorphic *Pnd* assays on the Pnd G7 clones are shown in Figure 5.9.
Figure 5.8  PCRs to Detect Xmv-9 Marker Genes in Fusion Cell Lines

Lanes 1-8: X9 B2 fusion cell lines

Figure 5.9  PCRs to Detect YAC Pnd Sequences in the 12 Pnd G7 Cell Lines
The same five clones of X9 B2 which were positive for both neo and the left side were also positive for Xmv-9, whereas the remaining three were not. Of the nine lines which were positive for Pnd, six had also been positive for the neo and left side sequences (see Figure 5.7). In other words, six cell lines appear to have integrated the entire Pnd G7 YAC, and five appeared to have integrated the entire X9 B2 YAC. This information is summarized in Table 5.2. These cell lines were frozen in liquid nitrogen. They were also prepared for the RT assays.

Table 5.2 Summary of Pnd G7 and X9 B2 Fusion Results

<table>
<thead>
<tr>
<th>X9 B2 (210kb)</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>✓</td>
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<tr>
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</tr>
<tr>
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</table>

<table>
<thead>
<tr>
<th>Pnd G7 (190kb)</th>
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<th>4</th>
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<th>9</th>
<th>10</th>
<th>11</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Neo gene</td>
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<td>x</td>
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</tr>
</tbody>
</table>

From this work, it was evident that L cells worked extremely well in the fusion experiments, and appeared to achieve a high frequency of complete integration of the YACs. This was important since this would not only allow YACs (large numbers, if necessary) to be efficiently screened, but would also ensure that Fv-1 could not be overlooked accidentally if integration was not complete. Table 5.2 shows a summary of the fusion results with L cells using X9 B2 and Pnd G7.
5.5.1 Virus Infectivity Assays

An RT assay was carried out on L cells (LM TK- and L-929 cells) and two of the newly fused cell lines from Pnd G7 and X9 B2. However, none of the cells was infected with virus. This experiment was performed to see if there was endogenous virus activity in the L cells. The possibility of virus production from uninfected L cells had been anticipated; the reason for this will be discussed. The results of this assay can be seen in Figure 5.10. These results clearly show that uninfected L cells were producing high levels of RT.

![Figure 5.10 RT Assay on Uninfected X9 B2 and Pnd G7 Cells](image)

The numbers indicate the volumes of cell supernatant harvested.
5.6 Devising an Alternative Virus Assay

Even though the L cells gave excellent fusion results, it now emerged that this cell line could not be assayed for virus susceptibility by RT activity. The 3T3 cell types used earlier gave incomplete and very low frequencies of fusion, so a further attempt at fusion using these lines was not considered worthwhile. The potentially large number of YACs which might have to be tested for $Fv-1^b$ activity was also taken into account.

An alternative virus infectivity assay was therefore required which did not rely on RT activity, but measured a characteristic unique to a newly infecting virus, for example, introduction of drug resistance into an infected cell. In addition, an assay was required which could avoid possible receptor-mediated interference from the endogenous virus; in other word, the use of an infecting virus which could use non-ecotropic receptors (see discussion).

Such an assay was developed by J.P. Stoye. N- and B-tropic viruses were constructed in which the envelope-coding region of the original ecotropic viruses used was substituted for that of the MCF virus (polytropic envelope). This ensured that these viruses would be able to infect L cells without the possibility of encountering receptor-mediated interference from the expressed endogenous ecotropic L cell virus. NB-tropic-MCF virus did not have to be constructed since such viruses occur naturally. An example (pMo-MCF-1) had already been made cloned (Bosselman et al, 1983). The MCF viruses were used as helpers to enable the introduction of a replication-deficient virus carrying the puromycin resistance gene. Because the helper viruses contain the $Fv-1$ determinant, introduction of puromycin would still subject to $Fv-1$ regulation. Infection with the puromycin resistance virus would enable infected cells to grow and form colonies in medium containing puromycin. Restriction of either N- or B-tropic MCF-puro virus would be manifest by a reduction in the number of puromycin-resistant colonies.
5.7 Control Assays

The NB-, N- and B-tropic MCF-puro virus stocks were tested to establish if they had comparable titres. This was to ensure that the \( Fv-1 \) assay would function correctly and any variation in colony number would be due to the action of \( Fv-1 \) and not the virus stock used. Since L cells were expected to inhibit B-tropic virus replication (see discussion), the 3T3FL cell line (\( Fv-1^{null} \)) was used for this purpose. 3T3FL cells were infected with varying volumes of stock virus. The volumes used for the NB-tropic virus were 20-fold lower than for the N- and B-tropic viruses because it was already known that the NB-tropic virus titres tended to be correspondingly higher. Figure 5.11 shows the assay using 3T3FL cells.

These data showed that the N- and B-tropic stocks had similar titres and that titres of NB-tropic virus were several fold higher. A prediction of the relative titres on 3T3FL and L cells could not be made with any degree of confidence because of the very different growth characteristics of the two cell types. This experiment, however, served as an indication of the amount of virus which might be suitable for the L cell assay. It was also important to have similar virus stock titres so any difference in levels of infectivity could be attributed solely to the action of \( Fv-1 \) activity in the L cells.
Figure 5.11 Control Fv-1 Colony Assay on 3T3FL Cells
5.8 *Fv-1* Assays on Pnd D11 and X9 J1 Fusion Cell Lines

At the time this first experiment took place, the NB-tropic MCF-puro virus stock was not yet available. G418-resistant cells were infected with 1000 μl of N- or B-tropic MCF-puro virus. Un-fused L cells infected with N- or B-tropic virus were used as controls. Figure 5.12 shows the results of this assay. Only one of the Pnd D11 assays is shown.

The L cell control demonstrated an approximate 10 fold restriction in the B-tropic titre compared to the N-tropic titre as revealed by the reduction in colony numbers. As expected, the L cells were *Fv-1^n*, based both on the typing of the Xmvs and Pnd and their genetic origin (see discussion). In this assay, therefore, *Fv-1* activity is manifest by only a 10 fold reduction in virus titre and not the 50-1000 fold observed in the RT assays. Therefore if *Fv-1^b* were introduced on a YAC, the N-tropic titre would be expected to show a similar reduction.

The X9 J1 cell line behaved exactly like the control, showing no reduction in N-tropic titre. In all three Pnd D11 lines however, the N-tropic virus appeared to have been restricted, almost to the level of the restricted B-tropic controls. This first assay therefore, demonstrated the possible presence of the *Fv-1^b* allele in the YAC Pnd D11.
Figure 5.12  *Fv-1* Colony Assays on Pnd D11 and X9 J1 Cell Lines
5.9 Confirming the Presence of the Fv-1\textsuperscript{b} Allele in Pnd D11

Further fusion experiments were set up to obtain new L cell lines containing Pnd D11. Many G418-resistant colonies were obtained of which fourteen were isolated and expanded. Before the PCR analysis was carried out, the first five cell lines picked at random were prepared for the Fv-1 assay. It was assumed, judging from previous fusion results, that at least one or two of these lines would have integrated the entire YAC. These cell lines were also frozen in liquid nitrogen in case of future need.

5.9.1 Problems With the L Cells

The cells were infected with the MCF-puro viruses as normal. However, cell colonies which appeared after several days of puromycin selection started to die for no apparent reason. The morphology of many of these cells was dramatically changed. Whereas healthy L cells were quite compact and dark in appearance, the dying cells were flattened, larger and more translucent, with what appeared to be highly enlarged nuclei. Figure 5.13 shows a comparison between a healthy colonies and these "sick" colonies. Similar changes have been observed by other workers, where the L cells have been described as having a "fried egg" morphology (C. Huxley, pers. comm.). Reasons for these disturbed growth characteristics are not known.

A mycoplasma test was performed on three of the cell cultures by NIMR media services, but no such contamination was detected. New virus stocks were prepared and fresh medium was ordered. A control assay was performed on un-fused L cells to determine if the problem had been solved. The result of this assay can be seen in Figure 5.14.
Figure 5.13 Healthy and “Sick” Puromycin-Resistant L Cells
Cells were infected with 50μl of NB-tropic virus or 1000 μl of N- or B-tropic virus. The B-tropic virus is restricted when compared to the N-tropic virus. The same volumes of virus were used in the subsequent assays.

The cells grew well, although in some colonies, a gradual change in morphology was observed after an extended period of growth, although this was not as severe as before. To reduce the effects of this problem, it was decided to fix the cells on the plate one or two days earlier than was normally the case. As a result, the fixed colonies were smaller than the optimum size, but the assay could nevertheless be unambiguously interpreted. With this new assay, a restrictive phenotype was seen as an approximate 20-fold reduction in colony formation, which was in fact twice the effect seen in earlier assays.
5.10 More Fv-1 Assays

PCR analysis for neo, the left side and Pnd, was carried out on DNA prepared from each of the 14 new Pnd D11 cell lines as before (see Figure 5.15).

![Image of PCR gel with bands labeled a1 a2 c d e f g h1 h2 i j k1 k2 l]

- Left side (200 bp)
- neo (130 bp)
- Poly Pnd (257 bp)
- + 128 and 129 bp for the YAC

Figure 5.15 PCR Assays on Pnd D11 L Cell Lines

Nine of the fourteen cell lines contained all three elements of the YAC and of the five which were initially selected for the assay, three (a1, c and d) were complete. Cells from the five selected colonies were revived from liquid nitrogen storage, as were fusion lines from the following YACs used in earlier experiments: Pnd G7, X9 B2 and X9 J1. The virus assay was repeated on all these cell lines. The results of assay can be seen in Figure 5.16. Of the six Pnd D11 lines tested, five of these restricted the replication of the N-tropic virus. None of the Pnd G7 or X9 YACs were able to confer this ability. It can be seen that in the case of cell line D11 (a2), N-tropic virus was restricted despite the absence of the left side and Pnd.
Figure 5.16  
*Fv-1 Assays on X9 and Pnd G7 Fusion Cell Lines*
Figure 5.17  *Fv-1* Assays on Pnd D11 Fusion Cell Lines
The modified assay was successful in the positional cloning of \textit{Fv-1} on a 190 kb YAC from the \textit{Pnd} locus. The single recombinant in the genetic mapping experiment, which separated \textit{Xmv-9}, \textit{Pnd} and \textit{Fv-1} from the other \textit{Xmvs}, proved extremely valuable in narrowing down the number of YACs which has to be tested. The nature of the ongoing and future work which will be required to isolate the actual gene encoding \textit{Fv-1} will be discussed.
Chapter 6
Discussion

In this project we set out to attempt the positional cloning of \( Fv-1 \), a mouse retroviral restriction gene. To go about this, the genetic map location of \( Fv-1 \) was refined, which placed marker genes \( Xmv-9 \) and \( Pnd \) within 1,200 kb of each other and \( Fv-1 \). YACs were isolated which contained these and other closely linked marker genes. An attempt was made to establish overlap between YACs from these loci by using PCR assays generated from the ends of several of the largest YACs. The assembly of a contig by this method was hampered by the presence of mouse repeat elements and a cluster of zinc finger genes.

YACs made from \( Fv-1^b \) DNA were introduced into mouse cells lacking this allele, by spheroplast fusion. The first two cell lines used, 3T3FL and NIH-3T3, gave poor fusion results with little or no fusion observed. Fusion cell lines which were recovered did not integrate the whole YAC. L cells, however, were found to give good frequencies of complete integration. The L cells used were found to produce RT activity which interfered with the \( Fv-1 \) assay. It was possible to devise an alternative assay, the use of which resulted in the identification of a YAC from the \( Pnd \) locus which encoded the \( b \) allele of \( Fv-1 \). This is the first reported case in which a YAC has been used for the positional cloning of a gene by virtue of its biological function.

6.1 The Genetic Mapping Studies

Four xenotropic murine leukaemia proviruses were known to map to the same region as \( Fv-1 \). To facilitate genetic mapping and cloning studies, unique PCR assays for the four elements were designed. Sequence
information was obtained from the 3’ LTR and into the DNA immediately flanking the proviral integration sites. This enabled the design of unique primers for each Xmv.

In previous mapping experiments (Frankel et al, 1989) the Xmvs were shown to lie in very close proximity to one another so there was a possibility that they may have arisen by gene duplication. Such a phenomenon has been observed with retroviral elements in the mouse Y chromosome (Eicher et al, 1989; Hutchison and Eicher, 1989). However, all the proviral flanking sequences were unique, which indicated that each integration event had occurred independently of the others.

The backcross yielded one particularly important piece of information. Before this work was undertaken, no recombinants had ever been observed between the four Xmvs, Pnd and Fv-1. However, a single animal (#322) out of a total of 368, showed a recombination separating Xmv-9, Pnd and Fv-1 from Xmv-8, 14 and 44 (see Table 4.5). It was this information which allowed the search for Fv-1 to be narrowed to the Pnd and Xmv-9 YACs. Statistical calculations which also took into account previous mapping data, suggested that Fv-1, Xmv-9 and Pnd would probably lie within 1,200 kb of each other (Stoye et al, 1994). This implied that relatively few YACs would be needed to ensure complete coverage of the area spanning all three loci. As stated above, constructing such a contig proved difficult. It was evident however, that if YACs containing these loci could be assayed for Fv-1 activity, regardless of whether or not they could be physically linked to each other in a contig, the chances of co-cloning Fv-1 in one of these YACs was quite high.
6.2 Screening the YAC Libraries

Screening the YAC libraries proved to be a relatively easy task, if somewhat time consuming with respect to sending results and receiving the next set of samples to be screened. The number of clones obtained was generally satisfactory from the Princeton and St Mary's libraries. However, even with an average of ~2.5 haploid genome equivalents contained in the libraries, the number of clones for each marker did vary considerably (from 0 to 8). Most of the Princeton and St Mary's YACs were stable (see Table 4.1). YAC X8 H10 occasionally gave rise to two separate YACs, even from the same clonal isolate, and Pnd C9 proved very difficult to isolate as a pure clone. Reasons for instability will be discussed below.

Finding a technique for preparing good quality high molecular weight YAC DNA for pulsed field gel electrophoresis was more difficult than anticipated. Although only one method is detailed here, others were tried which used spheroplasting enzymes other than lyticase (for example zymolase); these did not give consistent results. Also, methods using proteinase K, and other washes following production of the agarose blocks, were unnecessarily time consuming. The use of lyticase and the single LDS wash gave the most consistent and reliable results. No other particular problems in handling YACs, apart from instability, was encountered.

During the initial screening of the Princeton and St Mary's libraries, it became evident that no two marker genes appeared on the same YAC, which would have made linkage immediately possible. In view of the number and size-range of YACs isolated and the mapping data which places these markers within ~1 cM of each other, this result was rather disappointing. Although none of the YACs was found to have more than one marker, it was not unlikely that overlap exists between some of the YACs.
It was hoped that screening of the new large-insert Princeton library, which was made available at a later stage, would make contig assembly a simple matter. Unfortunately, all but two of the eight YACs found in the screen (X44 A9 and X9 C9) were highly unstable in the yeast host. Even those which were only mildly unstable could not be maintained in culture for any length of time. If, for example, a PCR-positive yeast colony was inoculated into selective medium and grown overnight, upon assaying individual clones from this culture, most were found to have lost their PCR marker. The fact that these clones were still able to grow in selective medium means that both the TRP and URA genes on the left and right vector arms of the YAC must have either stably integrated into the yeast genome, or the size of the YAC must have been reduced by deletions to such an extent that it was no longer detectable on pulsed field gels, i.e., less than ~30 kb.

6.3 Contig Building
Attempts were made to construct a contig by PCR analysis of all the available YACs. Initially, each YAC was assayed for the presence of any of the other marker genes which had been used to screen the libraries. The only YAC which contained more than one of the markers was X44 F1. This YAC contained Xmv-8, but was of no further use because of its extreme instability. Three large zinc finger-containing YACs were also made available. These Zfec YACs were derived from Fv-1a DNA which lack the Xmvs. DNA from an Fv-1a animal does, however, contain the sites into which the Fv-1b-associated proviruses integrated. To enable the Zfec YACs to be assayed for the presence or absence of the integration loci, new PCR primers were designed which allowed amplification of the DNA adjacent to the proviral integration sites. Some or all of these loci were indeed found in each of the Zfec YACs which was of considerable help towards building the contig.
6.3.1 "Bubble" PCR and Plasmid Rescue

The next task was to generate new PCR assays from sequence information derived from the very ends of YAC inserts to establish overlap with neighbouring clones. Two methods were used for this cloning YAC ends, "bubble" PCR and plasmid rescue. The first technique was attempted with four of the largest Pnd YACs and the largest most stable of the X44 YACs. In all cases, PCR products, varying in size from ~200-1500 bp, were generated from both the left and right sides of the YAC using one of the combinations of "bubble" and restriction enzyme.

The integrating plasmids pICL and pPLUS were also used, to rescue the ends of YACs X9 I11, X9 J1, Zn 5 and Zn 37. Again these were the largest YACs available screened for the particular marker genes and would perhaps maximize the probability of constructing a contig. This method also worked well in all cases, giving a size range of rescued insert of between 1-9 kb in length.

6.3.2 Comparison of the End-Rescue Methods

Despite the problems encountered with the YAC ends (see below), both rescue methods worked well. There were advantages and disadvantages to both. "Bubble" PCR is by far less work intensive than plasmid rescue and several independent samples can easily be processed at once. Also, YAC DNA from PFGE agarose blocks can be used, as opposed to the need for DNA from fresh YAC cultures transformed with pICL and pPLUS. The basic "bubble" method requires only a digestion, ligation and two rounds of PCR whereas plasmid rescue requires transformation of yeast followed by selection, PCR, digestion, ligation and rescue in E.coli. In addition "bubble" PCR can be performed on YACs from any yeast host, regardless of whether the yeast strain is rad52 or not.
The major advantage of plasmid rescue however, is that far larger fragments of DNA can be recovered. If repeat sequences are present at the YAC cloning site, "bubble" PCR may not provide enough DNA to allow the design of PCR primers; plasmid rescue is more likely to allow the identification of repeat free DNA. Obviously, if no such problems are encountered, then "bubble" PCR would be the method of choice.

The pICL and pLUS rescue of the St Mary's YACs was complicated by the need to transfer these YACs into a non-\textit{rad52} background to enable homologous recombination to take place. This was achieved by mating with a \textit{RAD52} wild type strain followed by sporulation and tetrad dissection and selection for the required genotype. Several manipulations of YACs require homologous recombination, so this type of transfer may be unavoidable. An alternative to tetrad dissection, which requires a dedicated dissection microscope, would be random spore analysis. This, as the name implies, requires only that the yeast by mated and sporulated. The sporulation mixture is separated, diluted and plated, from which individual haploid colonies grow. The colonies can then be analysed at random and the clones of the correct genotype selected. Obviously, the 2:2 segregation patterns of various genes and the YACs cannot be followed which could help to identify potential problems with, for example, multiple YACs.

6.3.3 Problems With YAC Ends

Problems arose upon sequencing the PCR products for the design of new PCR primers. Five of the ten ends isolated, using "bubble" PCR, showed homology to mouse B1 or LINE repeat elements. This was an extremely disappointing outcome, which caused severe difficulties in contig building. To make matters more difficult, upon testing the remaining five YAC ends for chimerism, it was found that only the left side PCR assays from Pnd D11
and Pnd C9 were of use. Two pairs contained unidentified repetitive DNA which obscured the chimerism test. The third primer pair, derived from the right side assay from Pnd C9, was able to amplify YAC DNA but was unable to amplify genomic DNA. An EcoR1 site was discovered within the segment of YAC insert which was amplified using these primers. It is possible, therefore, that this represented a co-ligation event of the partially EcoR1-digested genomic DNA during the construction of the YAC library. In other words this YAC was probably chimeric, i.e., DNA from two different regions of the genome had been joined to form a YAC. Obviously this meant that genomic DNA could never amplify with these primers. Co-ligation as a cause of chimerism has always been presumed but never demonstrated. The only other chimeric YAC, a 750 kb clone, ever to be characterized in detail, appears to have been formed as a result of in vivo homologous recombination between Alu repeats (Green et al, 1991b).

On analysis of plasmid-rescued X9 I11 sequences, it appeared that the same problems were occurring as had been the case when "bubble” PCR was used. The right side contained LINE repeat DNA and the left side PCR gave a non-chromosome 4 pattern implying that X9 I11 is chimeric. Once more, a potentially useful YAC (it being one of the largest available from the Xmv-9 locus) turned out to be unusable for contig building.

Analysis of X9 J1 proved to be even more surprising. Sequence from the right side of this YAC revealed what appeared to a zinc finger gene in the form of an 84-86 bp tandem repeat region, followed by a non-repeated region of at least several hundred base pairs showing similarly strong homology to a zinc finger gene. A non-coding, apparently unique region of DNA from the right side of X9 J1 was also sequenced. Much to our surprise we found almost identical zinc finger and non-coding sequence at the other end of X9
J1, over 300 kb away. Further sequencing of the two end clones of X9 J1 finally revealed non repeated sequences which were used for primer design.

The relative frequency of B1 and LINE repeats may have general implications for end-rescue experiments. LINE repeats contain conserved EcoR1 sites, as do many B1 repeats (Hastie, 1989). It is though that there may be as many as 1x10^5 LINE elements and 1.8x10^5 B1 repeats (Hastie, 1989) in the mouse genome.

The fact that in many cases, we found YACs with these repeats at their ends is therefore perhaps not entirely surprising. A rough calculation may help to illustrate this point: if the mammalian genome contains ~3x10^9 bp and an EcoR1 site occurs every 4096 bp (4^9) then there are a total of about 7x10^5 such sites in all. If each LINE (1x10^5) and B1(1.8x10^5) repeat is taken to have one EcoR1 site, then 2.8x10^5 EcoR1 sites will occur in these elements. So, a significant fraction of a YAC library with EcoR1 cloning sites contains ends belonging to one of these classes of repeat sequence.

6.4 An Area Of Instability Exists in This Region of Chromosome 4
In retrospect the first indication of an area of instability in the YAC clones became apparent when YAC Pnd C9 from the St Mary's library proved very difficult to isolate. Although the screening stages went well, upon streaking out the identified clone strain, fewer than ten colonies were isolated and of these, only two survived in liquid culture.

The next indication of an area of instability came when the new large-insert Pnd, Xmv-9 and Xmv-44 YACs were isolated. Without exception, all eight YACs showed some degree of instability, which varied from mild instability
to complete loss of the YAC (see Figure 4.7). It is also interesting to note that no YACs from the \textit{Pnd} locus could be isolated from the ICRF library. The smaller YACs from the Princeton and St Mary's libraries did not show any great degree of instability, probably for one of two reasons: 1) these YACs did not extend into the region of apparent instability because of their size, or 2) many of these YACs were by-products of larger unstable YACs which, having lost these areas of instability, had now become stable. If the latter reason is true, this would make contig building with these YACs extremely difficult.

A cause of instability could be that certain mammalian sequences may have biological activity which is detrimental to the yeast host and would lead to loss or alteration of the sequences (Vilageliu and Tyler-Smith, 1992). However, such a mechanism has not been demonstrated to date.

Instability problems have been reported by other workers. In one case, deletions were detected which gave rise to a succession of smaller YACs (Palmieri \textit{et al}, 1993). Just such a phenomenon was seen with YAC \textit{Pnd D11}, which was slightly unstable (see Figure 6.1). Although the identity of the offending sequences was not established, it was presumed that the cause was the interaction of internal recombinogenic sequences.

In another case, YACs containing tandemly repeated elements from the human \textit{Y} chromosome were isolated. In all cases the YACs were structurally unstable (Neil \textit{et al}, 1990). Such an explanation is not so readily available for the St Mary's YACs (\textit{Pnd C9}, for example) since the YAC host is recombination deficient and should not allow these events to take place at any great frequency.
Several YACs used in the human Y chromosome study were transferred to a *rad52* strain. This was found to improve the stability of some but not all YACs. It has been shown, however, that of YACs isolated from a region of instability around the *Zfx* gene on the mouse X chromosome, a St Mary’s clone was stable, whereas two others from the ICRF library were not (Hamvas *et al*, 1993). This locus encodes a zinc finger protein (Page *et al*, 1990).

Given the evidence of the Y chromosome tandem repeat elements, the instability of YACs at the *Zfx* locus and the instability of our YACs encompassing a zinc finger gene cluster, it is tempting to speculate that the reason for the instability in these YACs was due to zinc finger tandem repeats which the yeast host could not maintain. However, others (Bellefroid *et al*, 1993) have been able to isolate YACs containing clusters of zinc finger genes, so these *per se* do not cause instability. It is possible, of course, that the function of the individual zinc finger gene cluster may have a bearing on YAC stability.
6.5 Contig Assembly

Despite instability and interspersed repeat element problems, we attempted to assemble a rudimentary contig using primer pairs for both ends of X9 J1, Zn 5, Zn 37, the left end of Pnd C9 and the right end of Pnd D11 as well as the Xmvs. Further difficulties with repeats were immediately encountered. Both ends of X9 J1 and the Zfec YACs produced positive PCR reactions in well over half the YACs tested. The appearance of PCR products of different sizes, particularly in the X9 J1 reactions, was also surprising. Although the PCR reactions were designed with the assumption that they did not amplify repetitive sequences (as revealed by negative computer homology searches) all except the ends from the Pnd YACs appeared to do exactly that. This confusing set of results made it extremely difficult to assemble a contig with any degree of certainty.

A potential explanation for this problem comes from the presence of zinc finger protein genes in the region. It has been shown that zinc finger genes are often organised in large clusters (Crossley and Little, 1991; Bellefroid et al., 1993) and it has been estimated that there are up to 1000 of these genes in the mouse genome. They can encode from between 2 to 37 tandem finger repeats, often organised in a single exon. These clusters evidently span very large distances, perhaps hundreds of kilobases, as demonstrated by the assembly of a partial YAC contig of such a cluster (Bellefroid et al, 1993).

The zinc finger probe, Zfec23, which was cloned from a cDNA library by low stringency hybridisation (Crossley and Little, 1991), reacts with several genes co-segregating with the Xmvs in the BXD RI set (P. Little, pers. comm.). Our analysis of YAC X9 J1 implies that multiple copies of non-coding as well as coding sequences are present. Taken together these data suggest that a cluster of zinc finger genes has arisen by a process of local gene duplication in which
multiple copies of both introns and exons have been amplified. These genes are closely related to one another but different from the other clusters of zinc finger gene except in the finger region itself.

The benefit of carrying on with YAC end isolation was considered at this point. If the contig was to be further refined, the ends of other YACs would have to be isolated, with the additional task of making sure that they were repeat-free and not chimeric. It would also have probably been necessary to isolate new YACs to close any gaps and to span the area of instability, if this was indeed possible. Other methods which do not rely on such "sequence tagged sites" (Green et al, 1991a), such as generating PCR fingerprints using inter-repeat PCR, and partial restriction digest mapping using YAC vector probes, were attempted. Unfortunately this work was abandoned because it proved impossible to make reasonable sense of the data (not shown), which was possibly due to the regions of duplication. Alternatively, it may have been possible to sequence the polymorphic PCR products obtained with, for example, primers from the ends of X9 J1. This might have allowed particular polymorphisms to have been assigned to individual YACs.

Taking into account the amount of time which would be needed to improve the contig and the inherent risk of failure which this entailed, it was considered unwise to devote any more of the remaining time on this aspect of the project. Instead, a decision was made to rely on the genetic cross data and use those YACs which mapped closest to Fv-1 to try and co-clone the gene.
6.6 Introducing YACs into Mouse 3T3FL and NIH-3T3 Cells

Before YACs could be introduced into mouse cells, they had to be retrofitted with pRV1 which placed the neomycin resistance gene into the right arm of the YAC. They were then fused with mouse cells which were rendered G418-resistant if fusion was successful. The mouse 3T3FL cell line was used first in this experiment. This cell line was $Fv-1^{null}$ and was susceptible to both N- and B-tropic virus. It is not clear why this line has lost its ability to restrict virus. One possibility could be that the $Fv-1$ locus has been lost or mutated or perhaps the gene has been silenced by methylation. Methylation-induced silencing of non-essential genes appears a common feature of cultured mouse cells (Antequero et al, 1990). The possibility of a large-scale deletion would seem unlikely because the Xmv integration loci and $Pnd$ were found to be present (see Figure 5.2). An alternative explanation may be that a trans-acting mechanism has inactivated $Fv-1$ gene expression. Given this hypothesis, it may have been unwise, in retrospect, to use this cell line, since if $Fv-1$ had been introduced, it may also have been inactivated.

Frequencies of fusion with 3T3FL cells was extremely poor. After several attempts with different YACs, only two Pnd D11 fusion colonies were recovered. In neither of these did the entire YAC integrate, as demonstrated by the lack of left arm-specific sequences. An $Fv-1$ assay was carried out on these two lines, but no effect was observed on N-tropic titres. It is not known whether failure to detect $Fv-1^b$ activity was due to incomplete integration or because the gene, although present, was not being expressed.

Because of the poor results using 3T3FL cells, fusions were attempted with NIH-3T3 cells. During the course of retrofitting further YACs for these experiments it was discovered that YAC Pnd G7 was one of two YACs within the same yeast cell. Selection schemes following yeast transformation with pRV1, for example, which rely on the disruption of particular marker genes
in a YAC (in order to select for successful transformants) proved impossible in these cases. The same gene on the second YAC remained intact and thus the desired recombination event could not be detected. To resolve this difficulty, Pnd G7 was isolated way from its unidentified partner YAC by mating with a non-YAC containing yeast, followed by sporulation and tetrad dissection. Once separated, Pnd G7 was then able to be used for retrofitting and fusion as normal. How widespread the phenomenon of double YACs is unclear. This problem only seems to come to light when retrofitting experiments are attempted which rely on selection (Davies et al, 1992). It is possible that these double YACs occur as a result of co-transformation during library construction. An alternative method to remove an unwanted YAC is to transform the yeast with, say, pRV1, which disrupts the URA gene. At a certain frequency, the plasmid will integrate into the YAC of interest, but not into the unwanted YAC. 5-fluoro-orotic acid can then be used to select against the functional URA gene on the unwanted YAC, which is then lost from the yeast (Heikoop et al, 1994).

After several fusion attempts, only four colonies containing X9 B2 and only one from a Pnd G7 fusion were isolated. Once again, in no case was integration complete and the results of the virus assays suggested that the Fv-1b gene was not present in the region of the YACs which had integrated.

6.7 Problems With the 3T3FL and NIH-3T3 Cell Lines
The reasons why fusion was so poor are not clear. The only reported case of YAC fusion with 3T3 cells made use of a very small 75 kb YAC (Travner et al, 1989) and the type of 3T3 cell used was not specified. Three clones were recovered from 1 x 10^6 mouse cells used. The YAC used in this investigation were far larger, which may have reduced fusion frequency.
Workers using different cell lines have reported better success. Using Chinese hamster fibroblasts it was possible to recover 20 colonies from $1 \times 10^6$ cells (Riley et al, 1992) and using Chinese hamster ovary cells, 10 colonies were recovered although in this case the vector was a yeast plasmid (Ward et al, 1986). Other workers who have used L cells have reported equal, if not better success with levels up to 50 colonies per $1 \times 10^6$ mouse cells (Huxley et al, 1991; Pachnis et al, 1990). It is possible that L cells possess a nuclease activity which, by introducing a single break in the host DNA, allows YACs to integrate more easily than would otherwise be possible (V. Pachnis, pers. comm.).

6.8 Fusion Using L cells

Although the 3T3 cell lines were of the correct Fv-1 genotype to allow detection of integration of Princeton and St Mary's YACs and subsequent virus assay, their poor rates of fusion made their use impossible. Despite the risk that the L cells could harbour active endogenous viruses which might interfere with the Fv-1 assay (see below), we proposed to use them for fusion. Their reported success in fusion and the necessity to achieve complete integration at a reasonable frequency (which was apparently not possible with the 3T3 cell lines) made them the only choice immediately available. Fusions worked well, yielding many G418-resistant colonies with several different YACs. Over half of the recovered clones showed apparently complete integration of the YAC as judged by YAC arm and insert PCR assays. This frequency of fusion was approximately half that obtained by others (Pachnis et al, 1990), which may reflect the reliance of a selectable marker in the arm. In several of the reported above cases, the method of mouse cell selection relied either on genes contained within the YAC which complemented cell deficiency, or an antibiotic resistance gene introduced into the YAC insert. It
has been suggested that reliance on a selectable marker which has been inserted into one of the vector arms of the YAC (as in this work), as opposed to one in the insert, would lead to a much reduced frequency of fusion products that could meet the selection (Huxley and Gnirke, 1991). Of all clones analysed, it is interesting to note that in no case was the left side PCR detected without both the right arm and the marker gene being present. It would therefore appear that the left arm is detected only if complete integration has occurred.

An interesting phenomenon was the appearance of apparently healthy cell colonies after 10-12 days under G418 selection which then ceased to divide. This was seen also with the 3T3 cell fusions. As previously suggested (Pavan et al, 1990b) it is likely that these colonies were derived from transient neo\textsuperscript{r} transfections due to mitotically unstable unintegrated YAC DNA. By in situ hybridization studies it was observed that such extrachromosomal molecules were present for at least 27 cell divisions after fusion in half the cell lines recovered (Featherstone and Huxley, 1993). They were able to replicate extrachromosomally but segregated poorly. Alternatively it is possible that some YACs may have integrated into an essential gene which prevented sustained colony growth.

6.9 The Fv-1 Assay and the Nature of the L Cell Virus

Upon assaying the fused L cells, it was discovered that this cell line was producing its own reverse transcriptase activity from an expressed endogenous retrovirus which completely masked the Fv-1 assay results. It had been hoped that these cells would not be RT positive, since it has been demonstrated that not all L cell lines produce virus (Hall et al, 1967).
Reports characterizing the L cell virus are present in the literature (Fenyö et al, 1973). C3H (Fv-1\(^n\)) and C57BL (Fv-1\(^b\)) cells were infected with L cell virus. The results of an XC plaque assay indicated that the virus was able to replicate in C3H cells and could therefore be considered N-tropic. Positive results in the assay are normally seen by the formation of plaques in the XC cells. In this case however, plaques were not seen, but replication was detected only by the presence of occasional giant, multi-nucleate cell formation. This suggested that the virus could be defective and consequently might be undetectable by RT assay. This turned out not to be the case. Further characterization of the virus by sucrose density gradient sedimentation and the presence of RNA-dependent DNA polymerase showed that it was a reverse transcriptase positive retrovirus (Fenyö et al, 1980). The virus is therefore almost certainly a partially defective endogenous N-ecotropic MuLV.

6.10 The New Fv-1 Assay Detects Fv-1\(^b\) Activity in the YAC Pnd D11

An alternative Fv-1 assay was developed by J.P. Stoye which enabled virus activity to be measured by counting the formation of puromycin-resistant colonies after infection by N-, B- or NB-tropic viruses acting as helper to a defective virus carrying the puromycin resistance gene. The Pnd D11 fusion cell lines caused an approximate 10-fold restriction in N-tropic virus, which was comparable to the restriction of B-tropic virus caused by the Fv-1\(^n\) allele already present in L cells. This suggested that YAC Pnd D11 has introduced the Fv-1\(^b\) allele into the L cells. As expected, the NB-tropic virus was unaffected by the introduction of the Fv-1\(^b\) allele on the YAC.

After some unexplained difficulties with poor L cell growth, the assays were repeated on new Pnd D11 lines as well as several from other YACs. These results confirmed that Fv-1\(^b\) had been cloned on the 190 kb YAC, Pnd D11.
An interesting observation was made regarding the relationship between $Fv-1^b$-positive clones and the PCR analysis results on the fused cell line DNA. Despite apparently having only integrated $neo$ and not $Pnd$ or the left side, cell line Pnd D11 (a2) still had $Fv-1^b$ activity. This would suggest that $Fv-1^b$ lay between $Pnd$ and the right arm of the YAC.

6.11 Isolating the $Fv-1$ Gene

The first task in the immediate future is to isolate the $Fv-1$ gene itself. The most straightforward way is to isolate a cDNA encoding $Fv-1$. There are a number of ways that this may be approached. One method is the direct selection of cDNAs encoded by the YAC (Lovett et al, 1991; Parimoo et al, 1991; Parimoo et al, 1993). An entire cDNA library (blocked for repeats) is hybridized to gel purified YAC which has been immobilized on a filter. Non-specific cDNAs are washed away leaving the bound cDNAs to be eluted, amplified and cloned. It is also possible to carry out the reverse of this procedure and use YAC sequences as a probe for hybridizing to a cDNA library under conditions that reduce repeat hybridization. These approaches may present problems if the gene of interest is not highly expressed. This could well be the case with $Fv-1$ given that the gene product can easily be abrogated.

Alternatively, more indirect methods might be used. CpG islands mark the start of many genes (Bird, 1986; Lindsay and Bird, 1987). Using restriction enzymes with two CpG dinucleotides in the recognition sequence, it is possible to search for such islands. Probes derived from neighbouring regions would be suitable for screening cDNA libraries. A technique known as “exon trapping” has recently been developed and has been successfully used in our laboratory to trap exons from the mouse $Hairless$ gene (Cachon-Gonzalez et al, 1994). The DNA fragment of interest is cloned into an intron of a gene
contained in a vector. Splice sites flanking exons in genomic DNA are identified in transcripts and used in vivo to "trap" new exons into the vector-derived mRNA. Following synthesis of cDNA, the gene can then be amplified by PCR and sequenced (Buckler et al., 1991). This method has been shown to identify genes from mouse and human cosmid and genomic DNA in an expression independent manner (Church et al., 1994).

A cosmid library has been constructed from the YAC Pnd D11 to take advantage of one or more of these methods, since these smaller clones will be easier to manipulate and analyse. Cosmids were introduced into NIH-3T3 cells by lipofection. Cell lines from each transfection were assayed for \( Fv-1^b \) activity by the normal RT method. Several cosmids were identified which were \( Fv-1^b \)-positive. Currently, DNA fragments from one of the cosmids is being used in the "exon trapping" vector in an attempt to isolate and sequence the \( Fv-1^b \) coding region. So far, four potential exons have been cloned and sequenced.

### 6.12 Future Experiments

Once a full length cDNA has been isolated, its identity must be confirmed by reintroducing it into \( Fv-1^b \)-negative cells to make sure that it confers resistance to N-tropic MuLV. Fv-1 from n and nr tropic cells will be cloned. Allelic sequence differences in the gene, characteristic of particular \( Fv-1 \) alleles, should be present.

To investigate the models proposed for the mechanism of \( Fv-1 \) restriction it would be useful to discover the cellular sub-localization of the gene product and to what degree it is expressed. For this purpose, specific antisera to the gene product will be obtained. It would also be of interest to over-express \( Fv-1 \)
to see if retroviral restriction can be enhanced, in other words to force an increase the number of virus particles needed to produce a successful infection. The second model, described in the introduction, suggests that it may never be possible to entirely restrict the virus due to the theoretical possibility of it evading the \textit{Fv-1} product while mitosis is taking place, even if this does occur at a low frequency. On the other hand, if the \textit{Fv-1} product is soluble and ubiquitous in the cytoplasm, then the virus would almost never be able to evade \textit{Fv-1} if it were sufficiently highly expressed. However, it is conceivable that over expression might be detrimental to the cell or animal. It would also be useful to discover if \textit{Fv-1} plays another role in the cell. Since it appears to be constitutively expressed, it could have other important functions.

Other studies will involve solving the molecular interaction between the \textit{Fv-1} product and the viral determinant p30. The \textit{GAL4} di-hybrid system could be used to study the interaction and would allow the effect of defined mutations in the \textit{Fv-1} gene to be investigated. This will not only help to understand the mechanism of restriction, but if this gene directly affects viral integration, it may help to clarify the role of the nucleoprotein complex in this step.

Perhaps the most important task will be to clone the human homologue of \textit{Fv-1}. It is known that there is a large degree of synteny between the distal end of mouse chromosome 4 and the proximal end of human chromosome 1 (Abbott \textit{et al}, 1992; Bahary \textit{et al}, 1991). Specifically, the \textit{PND} locus has been mapped to human chromosome 1p36. It is highly probable that \textit{Fv-1} will be found here. This gene has potential implications in the treatment of retroviral disease. It may even be the case that the human homologue is active in reducing the incidence of viral-induced tumours/leukaemias and other cancers. No infectious examples of C type retroviruses have been
isolated in humans (Weiss, 1984) however, particles resembling C type viruses and other MuLV-related sequences have been found in large numbers (Martin et al, 1981; Repaske et al, 1983).

Other groups of retroviruses are known to cause disease in humans, however. The human T-cell leukaemia virus (HTLV) is now established as the causative agent of adult T-cell leukaemia-lymphoma, and acquired immunodeficiency syndrome is known to be caused by the human immunodeficiency virus (HIV). Obvious targets for the Fv-1 gene product, or an engineered alternative, are the HTLV and HIV gag-encoded proteins. A therapeutic strategy which prevents retroviral integration could be of great benefit.

The existence of Fv-1 was discovered over twenty years ago. In 1973, Frank Lilly and Theodore Pincus wrote that “the problem of the mechanism of action of the Fv-1 gene would seem to be a ripe one from the standpoint of the molecular biologist.” After only a slight delay, we now have the opportunity to discover how this gene functions. The impact of this information on the understanding and prevention of retrovirus infection will be potentially far-reaching.
References


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Appendix 1
Yeast Growth and Sporulation Media

YPD:
For routine growth of non-YAC containing yeast strains
Glucose 20 g/l
Yeast extract 10 g/l
Peptone 20 g/l
In water
For plates, add 20 g/l agar

Synthetic Dropout (SD):
This is a synthetic medium containing only the required nutrients for yeast growth. By omitting certain nutrients, YACs having particular markers can be selected. For example, to select for a YAC which has the TRP1 and URA3 genes in the left and right arms respectively, tryptophan and uracil are omitted from the medium.

Glucose 20 g/l
Yeast nitrogen base without amino acids 6.7 g/l
Adenine 20 µg/ml Arginine 40 µg/ml
Histidine 20 µg/ml Isoleucine 40 µg/ml
Leucine 60 µg/ml Lysine 80 µg/ml
Methionine 20 µg/ml Tyrosine 40 µg/ml
Valine 100 µg/ml Phenylalanine 50 µg/ml
Uracil 20 µg/ml
In water. For plates, add 20 g/l agar
aAHC
This is a rich, high adeneine medium deficient in uracil and tryptophan, and hence selects for both ends of a YAC. YACs will grow to almost the same density in a AHC as in YPD.

Glucose 20 g/l
Yeast nitrogen base w/o amino acids 6.7 g/l
Casein hydrolysate-acid (low salt) 10 g/l
Adenine hemi-sulphate (1%) 4 ml/l
In water
For plates, add 20 g/l agar

Sporulation Medium
Potassium acetate 20 g/l
In water
For plates, add 20 /l agar

Appendix 2
Yeast Strains

AB1380 MATa ade2-1 can1-100 lys2-1 trp1 ura3 his5 (pYAC4 host)
YPH857 MATa leu2-D1 trp1-D63 ura3-52 ade2-101 his3-D200 lys2-801 cyh2^R
CG379 MATa leu2-D1 ura3 his7 trp1
Appendix 3

PCRs Used for YAC Screening, Analysis and Genetic Mapping

<table>
<thead>
<tr>
<th>Origin</th>
<th>Oligo</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTR</td>
<td>SB 1</td>
<td>CCTCAGATTGATTGACTACC 3'</td>
</tr>
<tr>
<td>Xmv-8 flank</td>
<td>SB 2</td>
<td>GGCTGCTGTTATCTCTTTGG 3'</td>
</tr>
<tr>
<td>Xmv-9 flank</td>
<td>SB 3</td>
<td>ACTGACAGAGATGGAGTTCTC 3'</td>
</tr>
<tr>
<td>Xmv-14 flank</td>
<td>SB 4</td>
<td>TCCGACTCAAATGCCAGC 3'</td>
</tr>
<tr>
<td>Xmv-44 flank</td>
<td>SB 5</td>
<td>CTGAAGAGAAAGCGCTGTGG 3'</td>
</tr>
</tbody>
</table>

| Xmv-8 flank | SB 8 | GCTACAGGCAGTTTTATAGGC 3'              |
| Xmv-9 flank | SB 9 | TGGTATTCAGGTGGCAAGG 3'               |
| Xmv-14 flank| SB 10| ACAAGCATAGCCAACAGTCC 3'              |
| Xmv-44 flank| SB 11| ACATCTTTTGACTGCCTTCCC 3'            |

<table>
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<tr>
<th>Locus to be amplified</th>
<th>Oligos</th>
<th>Product size</th>
</tr>
</thead>
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<tr>
<td>Xmv-8</td>
<td>SB 1+2</td>
<td>297 bp</td>
</tr>
<tr>
<td>Xmv-9</td>
<td>SB 1+3</td>
<td>174 bp</td>
</tr>
<tr>
<td>Xmv-14</td>
<td>SB 1+4</td>
<td>259 bp</td>
</tr>
<tr>
<td>Xmv-44</td>
<td>SB 1+5</td>
<td>227 bp</td>
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</table>

| Xmv-8 flank           | SB2+8  | 257 bp       |
| Xmv-9 flank           | SB3+9  | 135 bp       |
| Xmv-14 flank          | SB4+10 | 186 bp       |
| Xmv-44 flank          | SB5+11 | 162 bp       |

The Pnd locus is amplified with SB 6+7 to give a 323 bp product.

SB 6    GAACCTGCTAGACGACCTGG 3'
SB 7    AAGCTGTCTGCAGCCTAGTCC 3'

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The polymorphic \( Pnd \) locus is amplified with Poly 1+2 to give a 257 bp product. The allele from C57BL/6J DNA \((Fv-1^b)\) can be cleaved by \( Pvu \) II to give 127 and 128 bp fragments. The C3H/HeJ allele cannot be cleaved.

Poly 1 \(\text{CCTGTTGCCAGGGAGAAAGA} \ 3'\)
Poly 2 \(\text{TTGCAGCGATAAAGGGATGG} \ 3'\)

Amplification conditions of all above primers: 1.5 mM MgCl2

- \(94^\circ C, 1\) min
- \(58^\circ C, 30\) sec \(\times 32\) cycles
- \(72^\circ C, 30\) sec

Primers used to detect YAC integration into mouse cells:

Following integration of the plasmid pRV1 into the right arm of the YAC to introduce the \( neo \) resistance gene, a \( neo \) sequence can be amplified to give a 130 bp product (primers neo 1 and neo 2). The left arm-specific assay amplifies a 204 bp product and are designed to amplify the junction between pBR322 sequence and the yeast \( SUP4 \) gene sequence (primers Left 1 and Left 2).

Amplification conditions are as above.

neo 1 \(\text{TGTGGATCAGCATGATCTGG} \ 3'\)
neo 2 \(\text{CCACCATGATATTCGGCAAG} \ 3'\)

Left 1 \(\text{GCTACTTGGAGCCACTATCG} \ 3'\)
Left 2 \(\text{AAAGTCTTGCCTAAACC} \ 3'\)
Mating Type PCR:

Three primers are added to a PCR reaction. \textit{MAT}a gives a 544 bp product, whereas a \textit{MAT}a gives a 404 bp product. A diploid yeast gives both products.

\textbf{AGTCACATCAAGATCGTTTATGG 3'}
corresponds to a sequence at the right of and directed towards the \textit{MAT} locus.

\textbf{GCACGGAATATGGGACTACTTCG 3'}
corresponds to a sequence within the \textit{\alpha}-specific \textit{MAT} DNA.

\textbf{ACTCCACTTCAAGTAAGAGTTTG 3'}
corresponds to a sequence within the \textit{\alpha}-specific \textit{MAT} DNA.

The MgCl\textsubscript{2} concentration is 2.0 mM, with the following cycling times:

\begin{itemize}
  \item 94°C, 1 minute
  \item 58°C, 45 sec \texttimes 32 cycles
  \item 72°C, 45 sec
\end{itemize}
Appendix 4
Primers and Oligonucleotides Used in
YAC End Rescue

“Bubble” PCR rescue of YAC ends:
The sequence of the “bubble” oligonucleotides and primer positions within
the YAC vector arms can be seen on the following pages. The PCR primers
used are listed below.

224 ("bubble" specific primer):
CGAATCGTAACCGTTCGTACGAGAATCGCT 3’

HYAC-C (left arm specific):
GCTACTTGGAGCCACTATCGACTACGCGAT 3’

LS-2 (left arm specific):
TCTCGGTAGCCAGTTGGTTTAAGG 3’

HYAC-D (right arm specific):
GGTGATGTCGGCGATATAGGCGCCAGCAAC 3’

RA-2 (right arm specific):
TCGAACGCACCGATCTCAAGATTAC 3’

The final MgCl2 concentration in all cases is 1.5 mM. The amplification
conditions were as follows:

\[
\begin{align*}
94^\circ\text{C}, 1\text{min} & \quad 94^\circ\text{C}, 45\text{sec} \\
62^\circ\text{C}, 45\text{sec} \times 1 \text{ cycle} & \quad 62^\circ\text{C}, 45\text{sec} \times 30 \text{ cycles} \\
72^\circ\text{C}, 45 \text{ sec} & \quad 72^\circ\text{C}, 45 \text{ sec}
\end{align*}
\]
Sequences Of The Oligonucleotides Used To Generate The "Bubbles" For "Bubble" PCR

Components of the \textit{Rsa I} bubble

\begin{itemize}
\item YAC insert digested with \textit{Rsa I}, \textit{Eco RV}, \textit{Hinc II} or \textit{Stu I}
\item Ligated to bubble
\end{itemize}

\begin{itemize}
\item Blunt end
\item \begin{align*}
5' & \text{GAAGGAGGAGAC} \\
3' & \text{CTTCTCTCTCTG}
\end{align*}
\end{itemize}

\begin{itemize}
\item Universal bottom
\item \begin{align*}
3' & \text{CTTCTCTCTCT} \\
5' & \text{GAGAAGGAGAGGAGAGAGAG}
\end{align*}
\end{itemize}

Components of the \textit{Hinf I} bubble

\begin{itemize}
\item YAC insert digested with \textit{Hinf I}
\item Ligated to bubble
\end{itemize}

\begin{itemize}
\item \begin{align*}
5' & \text{ANTGAAGGAGGAGAC} \\
3' & \text{CTTCTCTCTCTG}
\end{align*}
\end{itemize}

\begin{itemize}
\item Universal bottom
\item \begin{align*}
3' & \text{CTTCTCTCTCT} \\
5' & \text{GAGAAGGAGAGAGAGAGAGAG}
\end{align*}
\end{itemize}
pYAC4 Sequences Adjacent to the YAC Cloning Site
Showing Primer Positions Used in "Bubble" PCR

YIP5/pBR322 region

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YIP5/pBR322 region

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Primers used in plasmid rescue of YAC ends:

The following primers are used to verify the correct integration of the end rescue plasmids pICL and pLUS. pICL PCR generates an 870 bp product which can be cleaved by PstI into 702 and 165 bp products if the plasmid has been targeted correctly:

\[ \text{pICL 1} \quad \text{GCGCTTAATGCGCCGCTACAGGGCG} \quad 3' \]
\[ \text{pICL 2} \quad \text{GCTCACCGGCTCCAGATTTATCAGC} \quad 3' \]

Amplification conditions for pICL: 2.0 mM MgCl₂

\[ 94°C, 45 \text{ sec} \]
\[ 70°C, 1 \text{ min} \times 32 \text{ cycles} \]

The following primers amplify a 1855 bp product if the pLUS plasmid had targeted correctly:

\[ \text{pLUS 1} \quad \text{CTTGAGATCGGGCGTTCGACTCGC} \quad 3' \]
\[ \text{pLUS 2} \quad \text{TGAACGGTGTACCCCAACCGGAATTG} \quad 3' \]

Amplification conditions for pLUS: 2.0 mM MgCl₂

\[ 94°C, 45 \text{ sec} \]
\[ 70°C, 1 \text{ min 30 sec} \times 35 \text{ cycles} \]

(pLUS rescue sequencing primer: CAAGTCTGGGAAGTGAATGG 3')
Appendix 5

YAC Insert Sequences and PCR Primers

"Bubble" PCR Products (primers are underlined):

**Pnd G7 Left**: sequence from EcoRV/RsaI and RsaI/RsaI. Homology was found to a B1 repeat.

```
1  gaattcactc tgtagaccag gctagcatca aacctataga gatgccccctg
51  cccctgcccc tgcttcccca gttctgggtt caaagttgag catcaccac
101  cacaccacgc cacgtccata cccctgatcc actacagcac ccaagcatgt
151  aagaacagcgtg actggagata gatccagaa ccactgggggc ttctccagtc
201  tccaccatgc gaggactccct tgtccagtcc caatcaagcc catcagagct
251  cttgggccacc cacaagatct caacagggag taggggtctc caggat-3'
```

**Pnd G7 Right 1**: sequence from 1000 bp RsaI/RsaI. Homology was found to a LINE repeat.

```
1  aacctagtat aagtggtatat tagcccataaa gctcagatag accacgatac
51  aacctacagaa ccataagaga aggagatca aagtgtgat
101  gttctccagtc acttagaaaa Fggggagaaga ataatacaca ggggtagagag
151  ggtgagaga atctggggag gagagagag acgaggaag aagtagagac
201  gagagagag aatggagaata aatccataggt gccagaaat
251  cggagaaaa actgagcagtg gaaaatgggg-3'
```

**Pnd G7 Right 2**: sequence from 1000 bp RsaI/RsaI. The PCR assay failed the hybrid panel chimera test.

```
1  ggaattcactg aggagtagcact gaaatatttgg aaaaaggttc ccttctccct
51  tggaaacagtctg ctagtaacat aacagccttt gctcagtgac ctaagtctac
101  atacagact tgcctttgctt ctcctcattct cactgggcatt ctctgggtgt
151  ttctattaca gttcatgact tttaaataa taatctctc tctgtggctt
201  tctataaagag atctgagat tagataaat aatgataagat attctcttg
251  tttctttgtaa cacatttccc-3'
```

**Pnd D11 Left**: sequence from RsaI/RsaI Homology was found to a B1 repeat.

```
1  gaatttctaga aacagccagga ctagcagaaag gaacctttgta gttccagcct
51  gttgagaaggt caagcttgag ccaaatgttg agatgacttta cttggcttctt
101  gttaataccac gttgagcgtc tggaggtctg t-3'
```
Pnd D11 Right: sequence from StuI/RsaI. The PCR assay performed well.

```
1  ccttgcttta actccagccct cctggggtct aatgctctaga agcccttaac
51  tgcagcagaa agtgagcagc gatcacaaggg ttccgagaga agggctaggc
101  tgcagcagc acatagcaga ggaggctgcc ttcttagcat cttggggtta
151  aagccattga tgcagctttc aaggtctaca cacacagaaa aacactacaat
201  tctatgctaa tctatgcttt tctagagaga gccaacacca gaaataacaca
251  ggacaggtgc tttgtttta gtctctgttc c-3'
```

Pnd C6 Left: sequence from Rsal/RsaI. Possible B1 repeat homology was found. The PCR assay gave a smear.

```
1  gaattctagg ccaagccaaag cttacaccgtc gtagataatt gtctcagaaa
51  catgagttga gccagccggc tgtgtgctca gctcttttaat aacaaaaactc
101  tgggagcag aggcaagttg atctctgaaac tctgggccag tctaaacctc
151  gtaggtccca ggcagccgca ggcggctaat acagaaagat cctgtctcaac
201  cacccttaccc ccccatccgg aaaaatattaa aactgatgta gccatgaacc
251  agatatgacg gc-3'
```

Pnd C6 Right: sequence from HincII/RsaI. The PCR assay failed the hybrid panel chimera test.

```
1  aacaacagac cccgttagttt aatagtgcctca ctctggcttca tctcttttaa
51  taggtctcct cctagataaa gaagatgaaaa gctctgttgag atgagaagca
101  atgttaaaag cacagaagtt atctctgagaa gaggagcat ttaaggagaa
151  ctttagtctt atttgagtaa ggattgacat aagcatgttaa aagttgcattg
201  actgaaaccc tattgatagaa gactgtacgg agtgagctct c-3'
```

Pnd C9 Left: sequence from Rsal/RsaI. The PCR assay performed well.

```
1  accacacct gggctgacgca tataaaacc atcacaagga gattaagctt
51  gaaaaacctg gcttcagccag gtaatcagaa aacctacca ccaacctgacc
101  ttaagcagaa aacaatggggtg acgcattttc agccagcccc tgccc-3'
```

Pnd C9 Right: sequence from Rsal/RsaI. The PCR assay failed on genomic DNA.

```
1  actgtcttgg ttctatttttt gagttgtgaa ttctgttaatc aaaaaatcggg
51  tgtctgtgaca tgtgtggtggt tgtgtctggg ttcccatatt gatccattg
101  atgtacacaagt tgtctgtcct tgtgcctaat aacatgtagc tctgtgttc
151  aggattattt tagtcagctg ggtttttgtt attcccatatg aaggtgtttg
201  aatcgttac aatctgtgcc ac-3'
```
X44 A9 Left: sequence from \textit{Hinfl}/\textit{Hinfl}. Some B1 homology in second half of sequence. The PCR worked well on genomic DNA but failed the hybrid panel chimera test.

\begin{verbatim}
1   attttgtag tattatatt gaccttgtag ttatcagacg gaagttacaca
51  tagttcagt atttctatt attgtattg ggaagataat tcaacattgc
101 tgctagcata tagtttagct ttagaaaaag cttccattca gcatctatag
151 ccaaatcatc tccagttaag aatgtgcata tggttgataa tatttagatt
201 tgtattatc tattcttttt ccaagacagg atctcttctt gtagccttgg
251 cttctgtag acatactata tagacagcg ctagctcaaa tctagagata
301 tgctagatt t3'
\end{verbatim}

X44 A9 Right: sequence from \textit{HindII}/\textit{Rsal}. Some zinc finger repeat homology in second half. The PCR worked well on genomic DNA but failed the hybrid panel chimera panel test.

\begin{verbatim}
1   gacattatct aagattaataa catgtcagt gaagtttttcaaa aagttttcag
51  attcaatact cctagttaata gcagctaaac tggtaaaga aaatataacct
101 gttgatatca tattcaggcct ttggccagga gaacatatttt tcccataaaa
151 aatatatttac atatatttttt tcgagaataaa gaaagtttcag
201 catgtgattat atctgtagaga atctcttctt gtagataatta ctcttagat
251 tctagagata aggcaaaaat aaggaattca aacacactaat gtagattgaa
\end{verbatim}

Plasmid End Rescue Products:

Zn 5 pICL \textit{BamH1}-rescue: sequence from a 1.2kb \textit{EcoRI} fragment.

\begin{verbatim}
1   gaattctttaga gcattgcaacc acagaattgt tggtagcttac taaaagggcc
51  atacgatagta ttttttattgg aagttttttctggatattttt atatatgttt
101 tacagcata ctctagtgtt cctttgctaa aagttatacag acctcagcat
151 atgttagata ctctggcat tgttacataaa gcttttggattcctcataaa
201 tattatatca agatgtagcctaatagtagat caggtaaagaa ttgtttaagaa
251 aagatggaa atctctttta tt3'
\end{verbatim}

Zn 5 pICL \textit{SphI}-rescue: sequence from a 1.1kb \textit{SphI}/\textit{EcoRI} fragment. This goes back towards the sequence above. One primer at each end was used to give a 1000bp PCR product. PCR assay performed well.

\begin{verbatim}
1   gaattccatc aagagagata ccttgaatat aagttgtatg tgacaataac
51  aatcaactgtc cagttctgga aatcactagtt tttttataca gtagagaata
101 ttttataatg aagtgagac aacatccttt taccacagata tattcatctta
151 aaatcctaa gagaactacct cacagagaga aagctctcta tattcttgaag
201 ggtatcagct ctttaactct ggcctatctt ctagaatgtt aacagtgaat
251 acttacagga tacaacttt-3'
\end{verbatim}
Zn 5 pLUS XbaI-rescue: sequence from a 0.3kb EcoR1 fragment. PCR assay performed well.

1 gaaattcatac agtagagaaa acttacaaaa atgcagtgaa tgtgataaat
51 gcttttactaa taagggcaga ctggaattta atcgataat tttacatttt
101 aaaaaccc attaaagta tttttatgt gtaaatgca gtaccccaaa
151 catcagtcgct acaaatcaac agatatattca aacagtagag caacattata
201 aatgaaaatct atgagatagt cctttaaaca gaaatatcat cttaaatattc
251 actggagaat tcaag-3'

Zn 37 pICL SphI-rescue: sequence from a 0.7kb EcoR1-SphI fragment. PCR assay performed well.

1 gaaattcgtgaa agagatggag ttccagttga tcttgcttttt tccactgtgtg
51 tacatcagaa aagtttctag ccaagttggt ttcataaaca tattccaaaga
101 atgtgtgcca gtagacaaca aaggggttgg tgctttcaca aactgacatt
151 aagctgctcat tgtaataagac agggctgaca caaaacactg tatataaaa
201 gttccagatc gttcccaggaatcgaactct tattgcttggt gttctctatgg
251 aaggtgcttg aagtacaca agtggaatat atacacagaa ccaaataga
301 gttagatcta tattttctca gcaaatagct aggcagtatg tcagttattac
351 tggatacact atgt-3'

Zn 37 pLUS BamHI1-rescue: sequence from a 0.45kb EcoR1-EcoR1 fragment. The PCR assay performed well.

1 gaaattccaat atctggagacc actgcagatttt acactgaggc taataagtca
51 ggaagggcag aagattttag gtaagggtct gtagtcccca ggaatctagt
101 tccaaatctt aattacgctg aatactcatg gtgttctcag atttccaagt
151 gttcatacct atgtaacgac ttcaataata cagaaacgct gtttttaccc
201 atagacatgt ttgtactcttt ttagagatgctgtgaatttt cctttctatttt
251 tatctactat caacaaataa gagtaataga ctatataaata cacatataag
301 atccatcag atttgccagg gctctctggc acagagtaaat aatgaaaattg
351 accgtaatatt gataag-3'

X9 111 pICL BamHI1-rescue: sequence from a 2.3kb EcoR1-EcoR1 fragment. The PCR worked well on genomic DNA but failed the hybrid panel test.

1 gaaatccatca gttgattatgt ctgctccacgt tctgaagtggt ttgctctgtca
51 taagtgccaa tgacaaacta cttcattctgt gaggctgttaa accttgaaaa
101 actagactcg tttactttgg ggggggctgg cttaaaaact tttatcttca
151 caaccaaggt cagctattaa tattatagtg ttaacgcttt gattttttttc
201 cattttttt ttctatttga ttattttata gacacttctttttaaagcgatttga
251 acctctatgt tagtttttttt ttaattggag gttattcaaat tttgtctgac
301 tacttgaatg gctggatccaa ttacacaggag cttcgagaga tccaacgtag
351 t-3'
X9 J1 **plus** SacI-rescue: sequence from a 1.4kb SacI-EcoR1 fragment. Homology to LINE repeat.

```
1   gaattctctg tataagtttc tacctcatttt tttgattttg atgtttgttt
51  ttcttgttgt tagctctcttg atatcttttag atatatgtga tattagccct
101  ctctcaagtgg tgagagttgg gaaggggttt tttttctccc aatctgtgag
151  ttgcaccttt gtctatattg atatgtcatt ttgctgcaca aagctttctca
201  gtctctctttg tctcctctct aacttagagct ctaggctcatt
251  tgcaggtcttt ttaggaattt tctctctcttg aagtcagtt tgggctcttt
301  t-3'
```

X9 J1 **plus** SacI-rescue: direct sequencing from vector into the insert. A region containing two 84-86 bp tandem repeats, containing EcoR1 sites, were found which were homologous to a zinc finger gene. At least one more repeat unit was visible on the sequencing gel, but was not readable.

```
1   gaattctcag actgcccccc ca---start---gtaaagca tttgcccatat
41  tcactgcatt tggaaagttt ctcctcctgta gaaattctct gatgaataact
91  aagacnggat ttgctgg-end
107  ---start---gtaaagccat ttgctcatt cactgctttt gtaaggttttc
147  tctctcgttt gaaattctga tgaataactaa gattnggatt gttttgg-end
193  gaaaaa-3'
```

X9 J1 **plus** SacI-rescue: sequence from a 2.5kb EcoR1-EcoR1 fragment. This sequence follows on from that above in the same orientation. A partial tandem repeat is still present, but with many mutations and with no internal EcoR1 sites.

```
1   gaattccctg atgaataacta agatggtacct gatgggtaaa gcatttgctca
51  tttttctacta ttttgagtta ttttttccttt gataaatttt ctctgtgacct
101  ttttagtttt cattttatgtt tataatttt gtaaatatta ctctatgttt
151  aaggtctccc tgcataatttg tctttttccta cattgactttta tgtttagaaaa
201  caaaaatattt tct
```

X9 J1 **plus** SacI-rescue: sequence from the other end of the above fragment. Sequence has >95% similarity to that obtained from a pICL clone (see below).

```
1   gaattcccttt tactttttctt gagtgttctct aactatgtgct tataaatgtg
51  ctctgagata tccagagact ctctgagatg gaagattcata gttaaaaatca
101  taacttttcttt tttgtttttct tctttatact ttctgtttttc ttggaaaaag
151  taaccaaatgc tatatttcttt gaggct acctt atcttttttt acttagcttc
201  aqgtgaactta aaaaaagttca aagaaaaattt taataatttt caaacaagaaa
251  tattatttct gctacatata taatttctgt tttgggtttt atataaaaatat cttactatttt
301  cattaaatgt tatc
```
X9 J1 pLUS \textit{SacI}-rescue: sequence from a 0.9kb \textit{EcoRl}-\textit{SacI} fragment. The PCR assay performed well.

```
1  gaattccctgt catattctga aacctaattca agaacaggaa gtatgttattg
gttcctacta tggcttattg taaatgctct
tatagctagt tggtttctct tcctctagta aaggtgatag agttaatgga 
51  tctgaacatc cagcataatt tagacacctgt aatgtatatata tttgaggacct
101  acatccagga atatatatct atgaaacacttt ttttgataaa aagaaacatt
151  ccatggttac atgtagttta cagatagtaa agtgatggtt agtaaactgt
201  gctcca-3'
```

X9 J1 pICL \textit{BamH}I-rescue: sequence from a 1.0 kb \textit{EcoRl}-\textit{BamH}I fragment. This has \textless 95\% similarity to the pLUS \textit{SacI}-rescued 2.5kb \textit{EcoRl}-\textit{EcoRl} fragment and is in the same orientation.

```
1  gaattcattgt ttctttgagtt tttcctacta tggcttattg taaatgctct
gagatatc cagactctctg aatgagggaa attctagtt aatacataac 
101  ttattctattg ttgctctttt acattctctg gttctcttg gaaaagttac
151  ccctgattaga ttctctgagg tcactcctta ttctctatct agcttctatg
201  aagttaaaa agttcagaa aaatatttata atccagaac aagaaatatt
251  ttatctatat atataatattg tgcattttata aaatattctca tattgacatt
301  aatgttacc cttatattaag atgtaaata tacaagg-3'
```

X9 J1 pICL \textit{SacI}-rescue: sequence from a 1.1kb \textit{EcoRl}-\textit{EcoRl} fragment. Some zinc finger homology was found.

```
1  ttacccaga aatccccatgt tagtattcat cagaaattc atacaggaga
51  gaaaccttca aataagatttg tatgtgacaa atacaatact cacaagtcca
101  gtcttagaaa tcatcagttta ttctcataag tagagaatct ttataaatga
151  aqagttcaggg acaatctcttt accaagatat atcatctcaaa aacatctag
201  aqatactct aqagaacaa actttctaaa tatagttag gtatggaatcc
251  ttctcttttg gcctatttcc tagactgtta cagtcaatc ttacaggcta
301  gaa
```

X9 J1 pICL \textit{SacI}-rescue: sequence from a 1.2kb \textit{EcoRl}-\textit{SacI} fragment. The PCR assay performed well.

```
1  gaatttacagt tcagttttgta gcattaaaat gcattaataa atggtttttcag
51  ttaggtctct tgtgtttact aaatatcaagta agatctagtt caatgttcca
101  ggcaagtatt atctaggtta gataaagaga aacagaaata acgttagagc
151  ttccagcc tcagctcaaa gctgtgcttctcc tcttctagtta aggaggtat
201  gaaagaaacaa tcatcataatgaaac aataagtac gactgtctga cccaggtttcct
251  ggtagctat ccagacttgg tctgtgctgc atgtgtctga agttcatatt
301  tccattatt gacaacauagc c-3'
```
PCR primers generated from YAC ends:

PndG7 Right 2 (216 bp)  
CCTTGAAACATGTCTGCTAAC-3'  
ATGGTTACAAGAAACTAGAAG-3'

Pnd D11 Right (225 bp)  
CTTGCTTTAACTCCAGCCTC-3'  
CTCAGTAATAAGCATCATTTGC-3'

Pnd C6 Left (206 bp)  
AATTCTAGGCCAGCCAAGGC-3'  
AAGGGTGTTGAGACAGGATC-3'

Pnd C6 Right (228 bp)  
CTCAGTAATAAGCATCATTTGC-3'  
AATGCACACTCCGTACAGTC-3'

Pnd C9 Left (135 bp)  
ACCCTGGGCTGACATATAC-3'  
TGGAAACTGCTGACCCATTG-3'

Pnd C9 Right (214 bp)  
ACTGTCCTTTGCTATTGTG-3'  
CAAGATTACGGAATTCCAAC-3'

X44 A9 Left (254 bp)  
ACTTATGTGTTATCAGACGG-3'  
GTCTATATAGTATGTCTCAGG-3'

X44 A9 Right (255 bp)  
GATTGGAACATTGCAAGG-3'  
TTGGCCTATCTCTTAGAATG-3'

Zn 5 pICL (1000 bp)  
AGGAATGGCTGTCCTGATAG-3'  
AACGAGACAATCCATTACC-3'

Zn 5 pLUS (229 bp)  
TGCAATATGTGATAATGC-3'  
TTCTCCAGTGAATTTAAGATG-3'

Zn 37 pICL (226 bp)  
TCTGAAAGAGATGGAGTTCC-3'  
GAGTCAATGGTGCTTTGTGAC-3'

Zn 37 pLUS (240 bp)  
TCCAATATGTGACAGGACC-3'  
GAAGTCATGCAATTTAAGATC-3'

X9 J1 pLUS (243 bp)  
TGTCATGAGAATCATGACG-3'  
CATATGGGTCTTGTGTTAAC-3'

X9 J1 pICL (255 bp)  
GAATGAGTCTTTACTATCTC-3'  
GGAATTAATGACTTCAGAACC-3'

Amplification conditions as for the Xmvs
Appendix 6

DNA Size Markers (sizes are in base pairs):

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<th>λ (digested with HindIII)</th>
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