Molecular analysis of the Friend virus complex

Nicholas Hunt

B.Sc. (Hons.), Warwick

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Heinrich-Pette-Institut,

Hamburg

and

Department of Biological Sciences,

University of Warwick.

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Summary

This work was undertaken to molecularly clone the Mirand strain of the polycythaemia inducing Friend spleen focus forming virus (F-SFFVp) together with its replication competent helper virus, Friend murine leukaemia virus (F-MuLV). To this aim viral extrachromosomal DNA molecules (of both linear and circular nature corresponding to both of these viruses) which could be induced in Friend cell lines were molecularly characterised with respect to their quantitative increase during differentiation and subcellular location. One cell line, F4-6 in which considerable amounts of these extrachromosomal DNAs could be detected was utilised for the large scale production and purification of both SFFVp and F-MuLV extrachromosomal DNA molecules. Restriction enzyme analysis of such molecules in combination with Southern blotting enabled the construction of primary restriction enzyme maps which allowed the selection of a molecular cloning strategy. Both of these viruses were subsequently molecularly cloned and were subsequently shown to be biologically active after transfection into recipient cells.

A further aspect of this work was to construct a biologically active SFFVp containing a dominant selectable marker gene. A selectable construct was generated by joining different regions of the genome of the myeloproliferative sarcoma virus (MPSV) and SFFVp. A construct with the U3 region from the long terminal repeat (LTR) of SFFVp and the envelope gene region (gp55) of SFFVp (designated neo^R SFFVp) was found to be fully active as a selectable retroviral vector with identical biological properties to the wild type SFFVp. Neo^R SFFVp induced erythroid differentiation *in vivo*, with infected cells no longer requiring erythropoietin for differentiation. Furthermore neo^R SFFVp infected spleen cells could be used to generate immortal Friend leukaemia cells which were selectable with geneticin (neo^R).
A further construct, neo^R SFFV-M, which had a U3 region originating from MPSV was able to cause erythropoietin independent erythroid differentiation. However as compared to neo^R SFFVp and indeed the wild type SFFVp, neo^R SFFV-M was able to induce the erythroproliferative disease with a different kinetics. All attempts to isolate transformed Friend cells with this construct failed.

Finally, based on the neo^R SFFVp construct a retroviral vector with a deleted envelope gene (and a polylinker cloning site) was constructed in order to further examine the pathogenesis of SFFVp.

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Declaration

I hereby declare that this thesis has been composed by myself and has not been used in any previous application for a degree. All the work has been carried out by myself except for some of the colony assays in Chapter 3 of the results which were performed in collaboration with Dr. Christine Laker. I am also indebted to Dr. Jürgen Löhler for his technical assistance in the performance of the histological examinations.

Nicholas Hunt

List of abbreviations

ALV	avian leukosis virus
ASV	avian sarcoma virus
ATP	adenosine 5'triphosphate
bp	one base pair of DNA or RNA
BCIG	5-bromo-4-chloro-3-indolyl-β-D-galactoside
BFU-E	burst forming unit-erythroid
BSA	bovine serum albumin
CFU-E	colony forming unit-erythroid
CFU-M	colony forming unit-macrophage
CFU-S	colony forming unit-spleen
Ci	curie
CIP	calf intestinal phosphatase
cpm	counts per minute
CSF	colony stimulating factor
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddNTP	dideoxynucleotide triphosphate
dGTP	deoxyguanosine triphosphate
dpm	disintegrations per minute
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonulcease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetra-acetic acid

epo	erythropoietin
EtBr	ethidium bromide
FCS	foetal calf serum
FLC	Friend leukaemia cells
FV	Friend virus
GMEM	Glasgow's Modified Eagles Medium
GTU	Geneticin transfer unit
h	hours
HBS	HEPES buffered saline
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
IMDM	Iscove's Modified Dulbecco's Medium
IPTG	isopropylthio-β-D-galactoside
i.v.	intravenous
kb	one thousand nucleotides of DNA or RNA
kd	one thousand daltons
LTR	long terminal repeat
m.o.i	multiplicity of infection
min(s)	minute(s)
MOPS	3-(N-Morpholino) propanesulphonic acid
mRNA	messenger ribonucleic acid
MPSV	myeloproliferative sarcoma virus
Mo-MuLV	Moloney murine leukaemia virus
Mo-MuSV	Moloney murine sarcoma virus
Mu-CSF	multi-colonev stimulating factor
MuLV	murine leukaemia virus
neo^R	neomycin resistance
NP40	nonidet-P40

oligo dT	oligomeric deoxythymidine monophosphate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pers. commun.	personal communication
p.f.u.	plaque forming unit
p.d.i.	post DMSO induction
p.i.	post infection
PIPES	piperazine-N,N'-bis(2-ethane-sulphonic acid)
poly A	polymeric adenosine monophosphate
RNA	ribonucleic acid
rpm	revolution per minute
RSV	Rous sarcoma virus
rRNA	ribosomal RNA
sec(s)	second(s)
SDS	sodium dodecyl sulphate
SFFV	spleen focus forming virus
SFFVa	anaemia inducing SFFV strain
SFFVp	polycythaemia inducing SFFV strain
SM	phage storage buffer
TCA	trichloroacetic acid
TEMED	N,N,,N;N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) methylamine
UV	ultra violet
v	volume
w	weight

GENERAL INTRODUCTION

1. RETROVIRUSES.

a) History.

Members of the retrovirus family were originally recognised by their capacity to induce tumours in susceptible animals, and were the first infectious agents found to be oncogenic. The initial retrovirus isolate was described by Ellerman and Bang (1908), who transmitted a form of chicken leukaemia to previously uninfected birds by injecting them with a filtered bacteriologically free extract from leukaemic cells. This discovery of the first tumour virus was clouded at the time for leukaemia was not at that time recognised as a neoplastic disease. The demonstration of Peyton Rous (Rous, 1911), that a filterable agent (Rous sarcoma virus: RSV) isolated from the spontaneous sarcoma of a domestic chicken could produce sarcomas in other chickens clearly demonstrated that infectious agents could be oncogenic. Much later oncogenic retroviruses were also isolated from mammals, including the milk born mouse mammary tumour virus (MMTV) of mammary carcinomas in mice (Bittner, 1942) and the transmission of lymphomas/leukaemia by murine leukaemia virus (MuLV, Gross, 1951).

b) Viruses and cancer.

Despite the abundant evidence from laboratory studies for the oncogenic potential of these viruses, many workers remained somewhat sceptical about their role in naturally occuring cancers and experiments were considered only to be interesting *in vitro* phenomena, with no real appliance to *in vivo* tumourigenesis.

However further studies, and the improved methods for diagnosis and classification of tumours and the advent of new molecular techniques

for the detection and analysis of retroviruses changed this view dramatically. It is now clear that horizontally transmitted viruses (not only oncogenic retroviruses, but also oncogenic DNA tumour viruses) play important causative roles in a number of major cancers of animals and humans. Examples of DNA tumour viruses shown to be involved with malignancies include Epstein-Barr virus (EBV) which is associated with two human malignancies, Burkitts lymphoma and nasopharyngeal carcinoma (Henle et al., 1968; Epstein and Achong, 1979; Klein, 1983). Many adenovirus serotypes are highly oncogenic in a broad range of hosts with rodents being particularly susceptible (Trentin et al., 1962; Heubner et al., 1963; Rabson and Kirschstein, 1964; Gallimore et al., 1985).

Retroviruses also induce preleukaemia (leukosis) and lymphoid malignances in cats (Jarrett, 1984; Neil, 1985), cattle (Kettmann et al., 1975, 1976) and man (Poiesz et al., 1980, 1981; Gallo et al., 1983). Indeed they may also cause immune deficiency syndromes, in animals, with the best example being Acquired Immune Deficiency Syndrome (AIDS), that is caused by a human T-cell leukaemia virus (HTLV-III, HIV1), which belongs to a family of viruses (the human T-cell leukaemia viruses) which have also been shown to play roles in other neoplasias (Ratner et al., 1985).

c) Taxonomy of Retroviruses.

All retroviruses have common morphological, biochemical and physical properties that justify their inclusion into a single virus family, (Retroviridae).

i) Subfamilies.

Retroviruses are widely distributed in nature (Teich, 1982) and members of this family have been found in many vertebrate species, from birds to humans. There is also evidence for their presence in invertebrates (Heine et al., 1980; Shiba and Saigo, 1983).

By the Baltimore classification scheme (Baltimore, 1971) for viruses, the retroviruses are members of group VI as they have a single stranded RNA genome of positive polarity (i.e. it is the same sense as mRNA) and have a DNA intermediate during replication.

Retroviruses are divided into three subfamilies of unequal size; oncoviruses (Oncovirinae, oncos=tumour), lentiviruses, (Lentivirinae, lente=slow), and spumaviruses (Spumavirinae, spuma=foam).

a) Spumaviruses.

Spumaviruses have been isolated principally as contaminants of primary tissue culture cells (Hooks and Gibbs, 1975). These viruses derive their name from the characteristic "foamy" degeneration they induce in cultured cells. Spumaviruses have been found in a number of mammalian species. In feline species, feline syncytium-forming viruses (FSFV), have been isolated from both healthy cats (Hackett et al., 1970; Jarrett et al., 1974) as well as cats with a variety of non-neoplastic diseases (Ward and Pederson, 1969; Gaskin and Gillespie, 1972) and neoplasms (McKissick and Lamont, 1970; Hackett and Manning, 1971). Bovine syncytial virus (BSV) has also been isolated from normal cattle and cattle suffering from leukosis (Malmquist et al., 1969). There have also been several reports of foamy virus isolates from human tissues, with the first isolate coming from a patient with nasopharyngeal carcinoma (Achong et al., 1971; Epstein et al., 1974).

Another isolate has been isolated from the brain of a patient with dialysis encephalopathy (Cameron et al., 1978).

The first foamy virus isolate was of simian origin (simian foamy virus, SFV, Rustigion et al., 1955). There are now at least nine SFV serotypes being associated with Old World monkeys, New World monkeys, apes and prosimians (Johnston, 1961, 1971; Stiles et al., 1964; Rogers et al., 1967; Gajdusek et al., 1969; Hooks et al., 1972, 1973; Rabin et al., 1976). These viruses induce persistant infection but they have as yet not been associated with a distinct pathology.

b) Lentiviruses.

The two best characterised lentiviruses are visna and maedi viruses (Haase et al., 1978). These two nearly identical sheep viruses induce progressive neurological impairment and a chronic degeneration respectively, after an extremely long latent period ranging from several months to years. These viruses have been termed slow virus infections (Sigurdsson, 1954). Other closely related viruses have been isolated from sheep and goats, these include progressive pneumonia virus (PPV, Kennedy et al., 1968) and caprine encephalitisarthritis virus (CAEV, Cork et al., 1974).

c) Oncoviruses.

The oncoviruses form by far the largest subfamily of retroviruses and indeed may be further split into several genera and subgenera. They have been isolated from virtually all vertebrate species in which they have been diligently sought, including humans. Usually members of the subfamily are grouped initially according to the host species from which they have been isolated and by the morphogenesis of their

virions, (type A, B, C or D, as described below). Within these broad groups they are further classified by their host range, interference patterns, genetic content, antigenic relatedness, mode of transmission and pathogenecity. Despite their name, many oncoviruses have not been shown to induce disease in vivo.

The following discussion although generally applicable to all retroviruses, will focus mainly on specific examples of murine oncoviruses.

d) Criteria for retrovirus classification.

Retrovirus virions share a similar morphology (Teich, 1982). They are 80-130 nm in diameter and consist of an inner electron-dense core (nucleoid) that is surrounded by a lipid containing unit membrane. The core consists of ribonucleoprotein, surrounded by an icosahedral protein capsid. An inner coat core protein is located between the core and the envelope. The external surface of the envelope has glycoprotein "knobs" that are particularly prominant in the virions of certain retroviruses, (Bolognesi et al., 1978). The virion envelope forms as the virus buds from the plasma membrane of the cell.

In addition to these morphological similarities, the virions of all retroviruses share many physical, chemical and enzymatic properties. They are composed of about 60-70% protein, 30-40% lipid, 2-4% carbohydrate and 1% RNA, and have a buoyant density of 1.16-1.18 g/ml. Their infectivity is extremely sensitive to lipid solvents and detergents and to elevated temperatures (56°C for 30 min). Virions contain the enzyme reverse transcriptase in addition to the structural viral proteins.

The viral RNA genome is composed of two identical positive sense

single stranded RNAs that possess a capped 5' structure and polyadenylated 3' ends (as do most processed cellular mRNAs, Coffin, 1982). The two RNA subunits are bound non-covalently to each other near their 5' ends. Each RNA subunit is 3.5-9 kilobases (kb) depending on the virus. A specific cell-encoded tRNA that is essential for virus replication is non-covalently bound near the 5' end of the RNA, (Taylor, 1977). The presence of two viral RNA copies in retrovirus virions implies that these viruses are diploid.

The genomes of all replication competent retroviruses contain three genes encoding the structural proteins; gag codes for the internal structural proteins, pol codes for the reverse transcriptase as well as a proteinase and an endonuclease which have been assigned to this region, and env which codes for the envelope proteins. The order of these genes is the same in all retroviruses being; 5'-gag-pol-env-3'.

i) Virion morphology and morphogenesis.

The first systematic classification of retroviruses after their species of origin was based on variation in the electron microscopic appearance of the viral particles associated with different viruses. Four major types of retrovirus particles A, B, C and D are recognised and the viruses are subdivided by type in accord with their particle morphology (Fine and Schochetman, 1978). The vast majority of these viruses such as RSV and MuLV have the C-type morphology. These particles develop only as they bud from the plasma membrane, with the mature extracellular C-type particle being 80-110 nm in diameter, with a centrally positioned core within the envelope.

MMTV is the prototype B-type particle. These particles develop their inner core in the cytoplasm and acquire their envelope as they bud from

the plasma membrane. The mature particle which measures about 125 nm in diameter contain an eccentrically located electron-dense core.

Several primate viruses have been classified as type-D: their morphological development is similar to that of the B-type particles in that their inner core is assembled in the cytoplasm. However, the mature (budded) D-type particle resembles C-type particles more closely. They are more closely related genetically to the C-type particles, (Devare et al., 1978).

A-type particles are only found intracellularly and are devoid of infectivity. They do not bud from the membrane and lack a lipid containing outer envelope. There are two types of A-type particles: intracisternal and intracytoplasmic. The intracisternal forms are derived from endogenous viral genomes, that are apparently replication defective (Kuff et al., 1981). Little is known about their biology, except that they are expressed in early embryos and in murine myelomas and can reintegrate into new sites in the host DNA, (Kuff et al., 1983). The intracisternal A-type particles have been seen only in mice and a limited number of other mammalian species. At least some intracytoplasmic A-type particles are precursor forms of B- and D-type particles.

ii) Host range variants of murine retroviruses.

Another important concept of retrovirus classification is that of cell-tropism, i.e. defining properties of the virus and of those cells in which the virus will undergo a complete replication cycle. According to this scheme murine retroviruses can be classified into four different groups (which may be further grouped together as either endogenous or horizontally transmitted).

a) Ecotropic viruses.

These are viruses that will grow in cells of the species from which they were originally isolated, i.e. a mouse virus which replicates well in mouse cells, but poorly or at an undetectable level in cells of other species. Most ecotropic mouse retroviruses however replicate to a limited level in cells from other rodents, such as rats but are usually incapable of replicating in cells of higher mammals. The reason for this is that the required receptors which interact with the envelope protein are not present on the surface of these cells. Both endogenous (i.e. the MuLV strains occuring spontaneously in laboratory mice being transmitted as inherited genes from one generation to the next) and exogenous (i.e. those viruses that are not represented as integrated viral copies until after infection of the animal at a cellular level, which if then incorporated into the germ line may become a stably inherited endogenous virus) ecotropic viruses may be pathogenic.

Susceptible cells can be rendered resistant to specific retrovirus infection by pre-infection with a virus bearing the same (or very similar) viral env glycoprotein specificity. This phenomenon is known as virus interference and is caused by blocking of the cell receptor, (first described by Rubin, 1960). Ecotropic viruses use the same receptor on mouse cells and are thus subject to cross interference (Sarma et al., 1967) whereas ecotropic and amphotropic viruses use different receptors on mouse cells and ecotropic and xenotropic viruses use different receptors on rat cells (Bresmer and Baltimore, 1977).

b) Xenotropic viruses. (Levy, 1973).

These viruses are endogenous to one species but are unable to

replicate efficiently in that species, due to lack of the virus receptor. Most mouse cells lack receptors for xenotropic MuLV. However because of endogenous MuLV and thus expression of their *env* gene receptors may be occupied by this protein leading to a block at the receptor level. Activation of such viruses *in vivo* is not readily detected because the barrier to re-infection abrogates the establishment of a chronic virus producing state. They do however tend to have a broad host range on heterologous species. The majority of endogenous retroviruses display a xenotropic host range, although none of the xenotropic viruses have yet been shown to be pathogenic in any animal.

c) Amphotrophic viruses.

These viruses have a broad host range and replicate well in both the cells in which they were originally isolated and also heterologous cells including those from other species. They are horizontally transmitted and are not endogenous. An important criterion, which separates this group of viruses from ecotropic and xenotropic viruses is that they do not show cross interference or neutralisation (by antibodies directed against the envelope protein gp70) with these viruses. This is probably due to the fact that their *env* glycoproteins are unrelated to those of the ecotropic and xenotropic viruses. This group of viruses have not been shown to be pathogenic.

d) Dual or Polytropic viruses.

These are endogenous viral genomes present in mice which have recombined between ecotropic and xenotropic strains, that also, like the amphotropic viruses replicate well in both homologous and

heterologous cells. But, unlike the amphotropic viruses they are neutralised by antiserum to the major env glycoprotein, gp70, of both ecotropic and xenotropic retroviruses, and are also subject to cross interference with either of the two virus groups, although there are several classes of which some do not show cross interference. An example is mink cell focus-forming (MCF) virus so called because of their ability to induce focal growth or morphological alterations in monolayers of mink lung cells. (Hartley et al., 1977).

iii) Subdivisions of the ecotropic viruses.

The tropisms may be further sub-divided according to a particular mouse strain in which the ecotropic viruses are able to replicate. Inbred mouse strains have a genetic locus, designated Fv-1, that restricts replication of virus to varying extents (i.e. confers resistance or susceptiblity to the mouse, (Lilly and Pincus, 1973)). At this locus two alleles have been identified and denoted Fv-1^n and Fv-1^b, where n represents the prototype NIH-Swiss mice and b the prototype BALB/C mice, (Hartley et al., 1970; Pincus et al., 1971; Pincus et al., 1975). The mice carrying the Fv-1^n allele at both loci are designated Fv-1^{nn}, and are only infected by a class of naturally occuring murine leukaemia viruses. These are denoted N-tropic viruses and Fv-1^{nn} mice are resistant to another class of naturally occuring MuLV (B-tropic) viruses. Mice with the Fv-1^{bb} genotype show the reciprocal phenomenon. There is yet another type of ecotropic virus, the so called NB-tropic virus which is not regulated at the Fv-1 locus and is thus able to grow in both Fv-1^{nn}, Fv-1^{bb} and Fv-1^{nb} cells. These viruses are considered to be laboratory variants as they have never been isolated as naturally occuring viruses. Cell lines obtained from feral (i.e. SG1; Hartley and

Rowe, 1975) and random bred mice (3T3 Fl; Gisselbrecht et al., 1974) do not exhibit any Fv-1 restriction and have thus been designated a Fv-$1^{-/-}$ genotype.

The observations mentioned above are however not absolute and may be overcome by a high multiplicity of infection (as reviewed by Teich, 1982). As opposed to the receptor restriction, which appears to be absolute, the tropisms described above appear to be post penetration restrictive and have been mapped in or near the gag gene, (Schindler et al., 1977) and is indeed the p30 protein, (as described later, Gautsch et al., 1978; Trees et al., 1979).
This section is summarised in Figure 1.

iv) Transmission of retroviruses.

Retroviruses, like many other infectious agents may be transmitted from one host to another by contact between individuals other than from parent to offspring (so called horizontal transmission). However a more frequent mode of transmission is that from parent to offspring (vertical transmission). The second form may be subdivided into two mechanisms, i.e. congenital and genetic.

Congenital transmission or infection occurs when infectious viral particles released by the mother infect the offspring, of which the best known example is the milk born infection of newborn mice with MMTV in C3H mice (Bittner, 1942).

During genetic transmission the viral genome is vertically transmitted from one generation to the next, as a DNA provirus and is maintained as part of the genetic complement of the gametes, the so called germinal provirus. The germ-line transmission is not a particularly important mode of viral oncogenesis for it only applies to

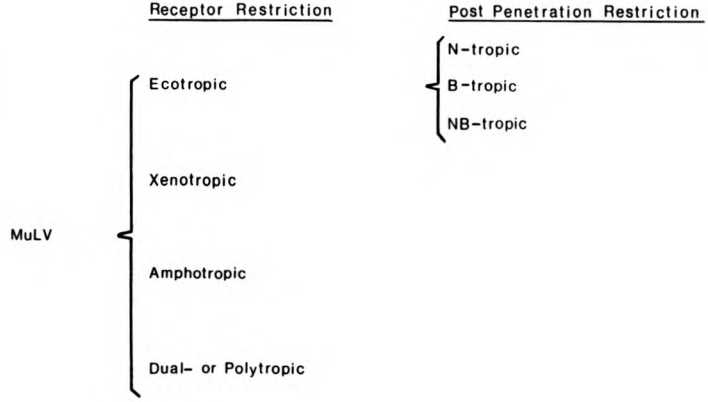

Receptor Restriction
Post Penetration Restriction
N-tropic
B-tropic
NB-tropic
Ecotropic
Xenotropic
MuLV
Amphotropic
Dual- or Polytropic

Figure 1. Classification of host range categories, demonstrated by C-type murine retroviruses

MuLVs are restricted at two levels, either at the receptor level, in which either the receptor for the respective virus is either present or not, or at a post penetration level in which the virus may attach and enter the cell but viral biosynthesis is inhibited.

particular inbred strain of mice, such as AKR mice, which have a high incidence of leukaemia due to their endogenous viruses, and GR mice which have a high incidence of mammary carcinomas due to endogenous MMTV.

e) Diseases caused by retroviruses.

i) Apathogenic infections.

Among the endogenous retroviruses it is a general rule that there is no pathogenicity. Only viruses resident in inbred strains of mice have high oncogenic potential, e.g. MuLV in AKR mice and MMTV in GR mice.

ii) Acute neoplasms.

Many C-type retroviruses are able to cause malignant tumours after only a very short incubation period, with one good example being the rapidly induced splenomegaly of the Friend virus complex where spleen enlargement is visible after 4 days.

Acutely oncogenic retroviruses induce specific kinds of leukaemias or solid tumours in animals and most of these viruses can also transform the same target cells after in vitro infection. These viruses carry specific transforming sequences called onc genes (Martin, 1970; Vogt, 1971; Bader, 1972; Baltimore, 1975), which usually occur as inserts substituting for portions of the viral structural genes thus rendering the virus defective for replication. The viral onc genes (v-onc) causing cell transformation are derived from cellullar (c-onc) genes which normally have a regulating role in the life cycle of the cell. However they exert an oncogenic effect when recombined into the viral genome under the influence of strong promoter signals provided by the

proviral long terminal repeat (LTR). Other acutely oncogenic viruses carry transforming genes derived from viral env genes, e.g. the spleen focus forming virus (SFFV) component of the Friend virus complex (see later).

iii) Slow neoplasms.

These are tumours induced by retroviruses that do not carry oncogenes as part of their genomes, which may be fast growing and highly malignant. Slow indicates the long latent period between infection and manifestation of the disease. However these viruses may also be subdivided depending on latency period. Friend-MuLV (Oliff et al., 1980) and Rauscher-MCF virus (Van Griensven and Vogt, 1980) are able to cause neoplasms in newborn mice within three to six months whereas Mo-MuLV results in a neoplastic disease only after an incubation period of longer than six months.

Various models of oncogenesis by the non-acute retroviruses have recently been reviewed by Neil and Forrest (1987).

iv) Anaemia, wasting and autoimmune disorders.

Many C-type viruses can cause anaemia, which may be aplastic, due to destruction of the stem cells in the bone marrow, hyperplastic due to a block in haematopoietic maturation (i.e. the anaemia strain of the Friend virus), or haemolytic as in several auto-immune syndromes associated with retroviruses.

There are also certain mouse strains that after infection with retroviruses (notably MuLVs) give rise to an auto-immune disease, i.e. infection of NZBxNZW F1 mice gives rise to an auto-immune disease that resembles systemic lupus erythematosis in man. (Talal and

Steinberg, 1974).

v) Neural diseases.

There is increasing evidence that various C-type viruses are also capable of causing neurological disorders, indeed a congenitally transmitted virus in feral Californian mice is able to cause both lymphomas and hind limb paralysis. The two diseases are not linked but both occur late in life, (Gardner et al., 1979).

In this brief overview (see Teich et al., 1982 for more detail), the terms acute and non-acute oncogenic retroviruses together with brief descriptions have been defined. Also it has been mentioned that there are defective (not replication competent) and non-defective retroviruses. Gene components of the latter may act in trans to rescue defective viruses thus forming a virus complex in which both viruses may be transmitted as infectious agents.

f) Morphological and biochemical features of retroviruses.

i) Genome RNA structure.

The descriptions given in this section are based on the non-acute, non-defective MuLV, which are made up of the classical retrovirus genes gag, pol, and env with no additional sequences such as onc genes. Other viruses will be introduced when appropriate for comparative purposes.

Retroviral genomes are relatively small and display a number of features in common:

a) They replicate through a proviral DNA intermediate, which subsequently integrates into the host cell genome, where it serves as a template for mRNA transcription, using the host cell's RNA synthetic

and processing systems.

b) They have very high recombination rates (in the order of 10^{-4}) with/between related viruses.

c) They have the ability to acquire new genetic material from the host cell genome which allows the virus to carry out neoplastic transformation.

d) They have close relationship with endogenous proviruses integrated in the cellular DNA of uninfected animals and which are passed from generation to generation by classical Mendelian inheritance.

Like any other viral genome, retroviruses contain nucleotide sequences with distinct functions, such as coding regions for specific viral proteins and recognition sites for the various enzyme systems involved in its replication and expression. Figure 2 shows these regions on a scale genome map of a prototype MuLV, Moloney MuLV, genome which was the first to be sequenced (Schinnick et al., 1981).

The dimer structure (60-70S structures) of the retrovirus genomes (Duesberg, 1968) first shown for RSV, posed many problems in the scientific community of the day, for diploidy in a virus was until then unknown. The genomic RNA subunits (34-38S) of retroviruses have several chemical modifications similar to those of eukaryotic mRNA, in that they have a poly(A) sequence of approximately 200 residues at the 3' terminus (Gillespie et al., 1972), with the exact 3' terminus being AOH. As in most eukaryotic mRNAs, the 5' terminus of each RNA molecule is modified with a typical capping group (CAP). The terminal virus-coded G residue (in the case of MuLV) is methylated at the 2' position of the ribose and 7-methyl GTP is added via a 5'-5'triphosphate linkage, giving the structure $m^7G5'ppp5'Gm$ (Furuichi et al., 1975; Keith and Fraenkel-Conrat, 1975).

THE GENOME OF MURINE LEUKAEMIA VIRUS

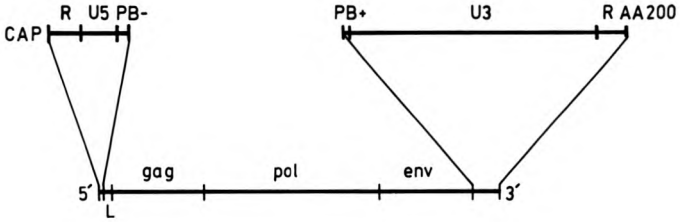


Figure 2. Structure of the non-defective Moloney-MuLV RNA genome

The map is drawn to scale and the terminal regions of the genome are based on their nucleotide sequences. The regions are defined and described in the text.

Even in highly purified virions, only about 50% (by weight) of the RNA is of viral origin. The remainder is composed of small species, most of which are of cell origin and which include variable amounts of 18S and 28S rRNAs as well as 5S and 7S RNAs identical with species found associated with ribosomes in uninfected cells (Bishop et al., 1970; Erikson et al., 1973; Walker et al., 1974).

Of the small 70S associated RNA molecules, the one most tightly associated with the genome is a single molecule per genome of tRNA which has been identified as a primer for DNA synthesis. In the case of MuLVs this tRNA appears to be $tRNA^{Pro}$ (Peters et al., 1977). tRNA molecules are associated with the genomic RNA by base pairing of the 3'-terminal 16-19 nucleotides of the primer (Peters et al., 1977), with complementary sequences in the genome referred to as the primer binding site PB(-), which is located 100-200 nucleotides from the 5' end of the genome.

ii) The redundant sequences (R).

This is a short sequence 20-80 nucleotides in length, depending on the virus, which is repeated at both ends of the genome and is presumed used during replication to permit the transfer of the nascent DNA chain and DNA polymerase from the 5' end to the 3' end of the genome (Joho et al., 1978). This process will be described in more detail later, see replication.

iii) The U5 region.

This is defined as the 80-100 nucleotide region of unique sequence which separates R from the primer binding site PB(-) and has up until now been assigned no functional role, although this sequence (together

with the R sequences) is highly conserved among related viruses. Furthermore the predicted conformational structure in this region of the genome is conserved (Devare et al., 1982).

iv) The primer binding site PB(-).

This is the binding site of the tRNA primer for negative strand DNA synthesis.

v) The leader region, L.

This is an untranslated region between PB(-) and the initiation codon for gag and has two functions relevant to the replication cycle. In MuLV-related viruses, as well as MMTV, this region contains the splice donor for env mRNA, whereas in ALV related viruses it is found directly following the sixth codon of gag.

The second function performed by the L region is to provide a signal for packaging, as indicated by deletion mutants of ALV and MuLV related viruses (Mann et al., 1983; Watanabe and Temin, 1983). The L region of many MuLV related viruses also contains an open reading frame preceding the initiation codon for gag which apparently acts as a signal sequence for the glycosylated version of the gag precursor, gPr80^{gag} (for explanation see; biosynthesis of viral proteins).

vi) The coding regions.

The assignment of the various gene products to the viral genome and also their position in the virus particle is shown in Figure 3.

a) The gag-pol region.

The gag gene of all retroviruses analysed have an overall structural

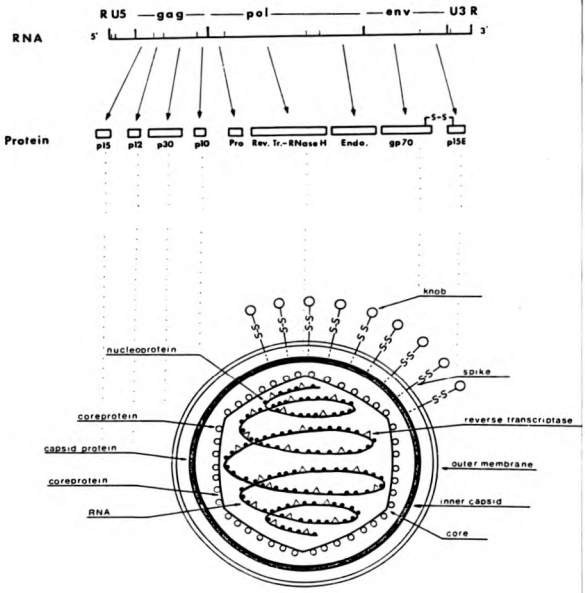

R U5
gag
pol
env
U3 R
RNA
5'
3'
Protein
p15
p12
p30
p10
Pro
Rev. Tr.–RNase H
Endo.
gp70
S-S
p15E
knob
spike
nucleoprotein
coreprotein
capsid protein
coreprotein
RNA
reverse transcriptase
outer membrane
inner capsid
core

Figure 1. Schematic representation of the arrangement of the structural proteins and their corresponding gene location, within the virion

This figure depicts the genome organisation of a type C retrovirus with the corresponding protein products and how they are thought to be arranged within the virus particle.

Adapted from Dickson et al., 1984.

similarity and are conserved (at the amino acid level) within each group but no sequence homology (at the nucleic acid level) is discernable between groups. There is a detectable nucleotide and amino acid sequence relationship between the gag gene of MuLV and simian sarcoma virus (SSV) for example but not between MuLV and RSV. These two genes are grouped together here for both the gag and pol gene products are synthesized as a simple high molecular weight precursor polyprotein (either the Pr65gag or the Pr200 gag-pol) which is subsequently cleaved to give rise to the mature proteins. Cleavage of this polyprotein results in the formation of the pol (NH_2-p14-p80-p46-COOH) precursor which is then further cleaved to give rise to the mature proteins (Figure 4). Since only genomic sized RNA (and not the spliced subgenomic RNA which was mapped to the 3'end of the genome) is able to direct the synthesis of gag and gag-pol precursors in vitro (von der Helm and Duesberg, 1975; Phillipsen et al., 1978) and in vivo (Fan and Verma, 1978) it was thus established that these genes must occupy the 5' half of the genome. However the idea that pol is expressed via an approximately genome-sized mRNA spliced to remove the terminator that ordinarily generates the carboxy terminal of gag is coming more and more into decline, for no one has yet shown the presence of such an RNA. More recent evidence from the sequence of the translation products of the MuLV genome, in which cleavage of the Pr200gag-pol, which generates the amino terminus of the p14 protease, takes place at a position corresponding to the fourth amino acid prior to the carboxy terminus of Pr65gag. The amino-terminal amino acid sequence of p14 reveals a precise match to the gag and pol amino acids predicted for the nucleotide sequence surrounding the UAG terminator. Furthermore, at the position corresponding to the terminator a

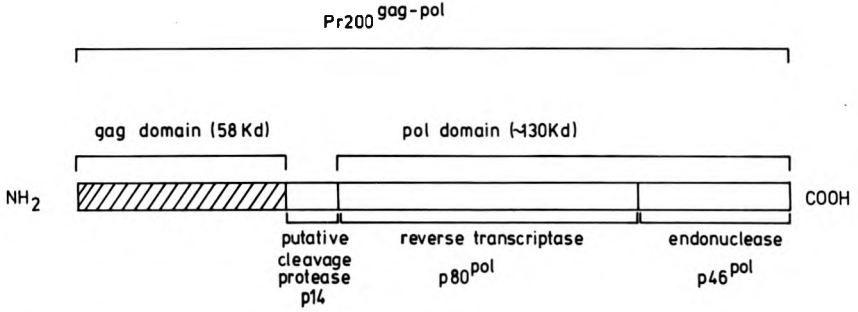

Pr200 gag-pol
gag domain (58 Kd)
pol domain (~130Kd)
NH2
COOH
putative cleavage protease p14
reverse transcriptase
p80 pol
endonuclease
p46 pol
NH2
COOH
p15 - pp12 - p30 - p10
Pr65 gag

Figure 4. The predicted structure of Moloney-MuLV Pr200$^{gag-pol}$

The scale diagram shows the location of gag and pol specific peptides within the Pr200$^{gag-pol}$ precursor. The gag specific regions are indicated by the hatched region and the pol specific region by the open box.

glutamine residue has been inserted. Thus the gag-pol fusion protein must have been generated by suppression of termination, i.e. by misincorporation of a glutamine (Yoshinaka et al., 1985).

The observation of Faller and Hopkins (1978) that p30^{env} contained a sequence involved in abrogating Fv-1 mediated cell resistance to MuLV infection was based on T1 oligonucleotide maps and protein analysis of biologically generated recombinant viruses. This conclusion has been confirmed and extended by experiments utilising site-specific recombinants generated from cloned MuLV DNA (Boone et al., 1983; Des Groseillers and Jolicoeur, 1983; Ou et al., 1983). In all cases the difference between N- and B-tropic viruses mapped to the two amino acid regions encoded by bases 1605-1610 of the AKR-MuLV genome (1590-1595 in Moloney MuLV) corresponding to amino acids 109-110 of p30^{env}.

The pol gene apparently encodes two or three polypeptides depending on the virus, in the 5'-3' order, protease-polymerase-endonuclease. Nucleotide sequence of a number of polymerase genes has revealed that pol is the most highly conserved part of the retroviral genome and is the only region for which detectable homology at the amino acid level is seen in viruses from different groups.

b) The env gene.

The env gene encodes the surface proteins of the virion that are required for recognition of specific cell surface receptors necessary for initiating infection. Because of their location and role, the env products determine both host range and the neutralisation antigens of the virion. Although not closely related to one another the env genes of different retroviral groups show a great deal of structural similarity. There is an overlap between pol and env such that the

splice acceptor delineating the 5' end of env precedes the 3' end of pol and translation of env is arranged so that the amino terminus of the primary product is within nucleotide sequences also translated to give the carboxyl terminus of the pol product, although in a different reading frame. The 5' terminal 30-100 codons of env encode a signal peptide cleaved off as a consequence of transmembrane processing of the env precursor. The resulting glycosylated precursor is cleaved into two proteins which remain associated via disulphide bridges. The larger (340-450 amino acids) aminoterminal protein contains determinants specifying host range. The smaller (180-230 amino acids) carboxyterminal protein always contains, near its carboxy terminus a hydrophobic domain of 23 amino acids or more, presumably constituting a transmembrane anchor region, followed by a basic amino acid and a small (approximately 20 amino acids) cytoplasmic domain which could be involved in recognition of capsid proteins.

vii) The 3' region.

The primer binding site, PB(+): This has been formally defined as the region 5' to the initiation site for positive strand DNA synthesis, although the precise primer binding sequence for this region is somewhat variable. This region is rich in purines and shows a high degree of sequence conservation among otherwise unrelated viruses.

viii) The U3 region.

The U3 portion of the genome forms the bulk of the LTR in the provirus and contains a number of short sequences near its termini that provide signals for integration, initiation and in some viruses polyadenylation (e.g. MMTV, BTLV). The function and organisation of

these signals will be discussed in detail in the following description of replication.

g) Replication.

Retroviruses are unified in their method of replication, in that they follow the cycle RNA-DNA-RNA-protein.

The replication cycle may be split into two phases.

1) Steps leading to the synthesis and integration of the viral DNA, from a copy of the viral RNA. These steps include, attachment to and penetration of the cell, priming and polymerisation of both strands of viral DNA leading to production of linear and circular forms of viral DNA and insertion of the viral DNA into the host chromosomal DNA.

2) Expression of the viral genes. This includes transcription of the viral RNA, translation, modification of the viral proteins, assembly of viral particles and finally maturation from the host cell. An overview is presented in Figure 5.

i) The provirus hypothesis.

Up until 1970, the hypothesis based on work with RSV (Temin, 1964) that retroviruses replicate via a DNA intermediate (the provirus) was not generally accepted. Most of the evidence was indirect and came from experiments using inhibitors of DNA synthesis such as actinomycin D, which interfered also with retrovirus replication. At the time replication of all known RNA viruses was unaffected by such inhibitors (Temin, 1963, 1967; Bader, 1965). However experiments using such inhibitors confirmed the provirus hypothesis (Boettiger and Temin, 1970; Balduzzi and Morgan, 1970; Duesberg and Vogt, 1969). The deciding observation came in 1970 when Baltimore (1970) and Temin and Mizutani

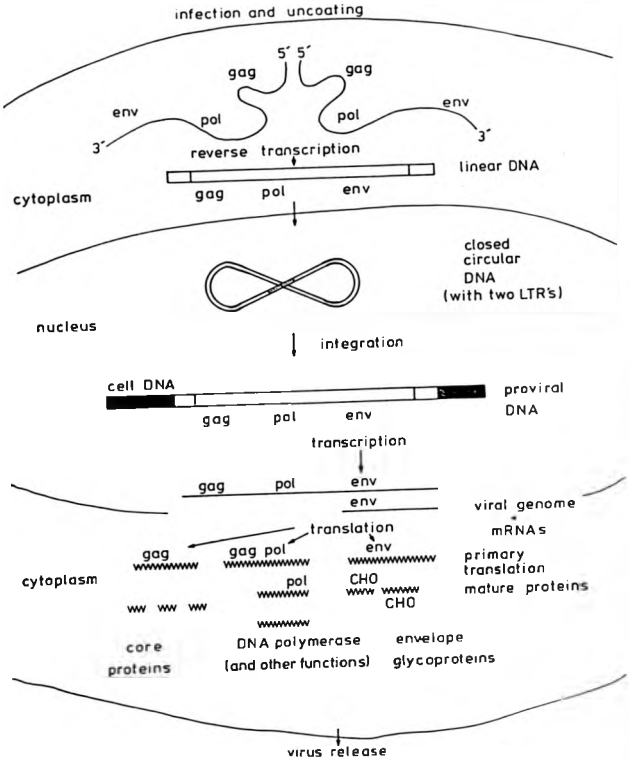

infection and uncoating
5′ 5′
gag
gag
env
pol
pol
env
3′
3′
reverse transcription
linear DNA
cytoplasm
gag
pol
env
closed
circular
DNA
(with two LTR's)
nucleus
integration
cell DNA
proviral
DNA
gag
pol
env
transcription
gag
pol
env
env
viral genome
mRNAs
translation
gag
gag pol
env
primary
translation
cytoplasm
pol
CHO
mature proteins
CHO
core
proteins
DNA polymerase
(and other functions)
envelope
glycoproteins
virus release

Figure 5. Schematic representation of the retroviral life cycle

The processes shown represent a complete retrovirus life cycle of a non-defective MuLV in which the proviral DNA is integrated such that the viral genes may be expressed and thus give rise to infectious viral progeny.

The life cycle is divided into cytoplasmic and nuclear phases.

(1970), independently demonstrated the presence of a RNA-directed DNA polymerase activity in purified retrovirus virions which provided the necessary biochemical basis for the provirus hypothesis. Further support came from the demonstration that retrovirus RNA hybridised specifically to infected cells and that DNA from infected cells was infectious (Baluda and Nayak, 1970; Hill and Hillova, 1972).

ii) The infection process.

Attachment and uptake: That the virus binds to specific receptors on the cell surface and that this is specific has been discussed earlier.

The process of uptake of virus is not well understood, but it has been proposed that the internalisation of retroviruses in fibroblasts occurs by endocytosis and that the viruses enter and infect cells through lysosomes or other acidic vesicles. Conceivably uncoating and transfer from the acidic vesicles then occurs by a low pH-dependent membrane fusion. This is supported by two sets of observations, in that the virus particles are rapidly degraded and that infection is inhibited by lysomotropic substances (e.g. chloroquine, amantadine, tributylamine, methylamine and ammonia) at a step shortly after internalisation (Anderson and Nexo, 1983). If the retroviruses behave like better studied viruses such as semliki forest virus (SFV), then one could imagine that membrane fusion must occur between the virion membrane and the endosome membrane, delivering the virion core into the cytoplasm of the cell (White et al., 1983).

iii) The synthesis of viral DNA.

Within a few hours of infection the core of the virion begins to carry out the process of reverse transcription of the viral RNA. This

is depicted diagramatically in Figure 6.

All replication competent retroviruses carry a gene (pol) which is capable of transcribing RNA into DNA (RNA-directed DNA polymerase, reverse transcriptase or deoxynucleoside triphosphate; DNA deoxynucleotidyl transferase). This molecule is present in the mature virion. Initial DNA synthesis from the RNA template gives rise to a single strand of DNA which is called the minus strand, since virion RNA is the same sense as mRNA which by definition is plus sense. This minus sense DNA is synthesized as a continuous piece of DNA, however its synthesis in practice is performed in three discrete steps because this strand is synthesized from three distinct templates. This minus strand DNA then acts as a template for the synthesis of complementary (plus strand) DNA, thus leading to a linear double stranded DNA molecule. These steps are discussed in more detail in the following sections.

DNA synthesis begins with extension of the paired hydroxyl terminus of the primer tRNA, which is located to the right of the U5 boundary (Figure 6.1). When the elongating minus strand reaches the 5' end of the RNA template it stops giving rise to the so called strong stop DNA (which is easily seen both in vitro and in vivo). Thus to continue with the minus strand synthesis the polymerase and the strong stop DNA complex must jump from the 5' to the 3' end of the virion RNA, a jump which is facilitated by the R sequences found duplicated at both ends of the RNA and the inherent RNAse H activity of reverse transcriptase. This enzymatic activity degrades the R sequences at the 5' end of the RNA (Figure 6.2), which enables the minus strand DNA that is complementary to these sequences to base pair with the R sequences located at the 3' end of the viral RNA (Figure 6.3). It is however not clear whether the jump is selectively made to the 3' end of the same

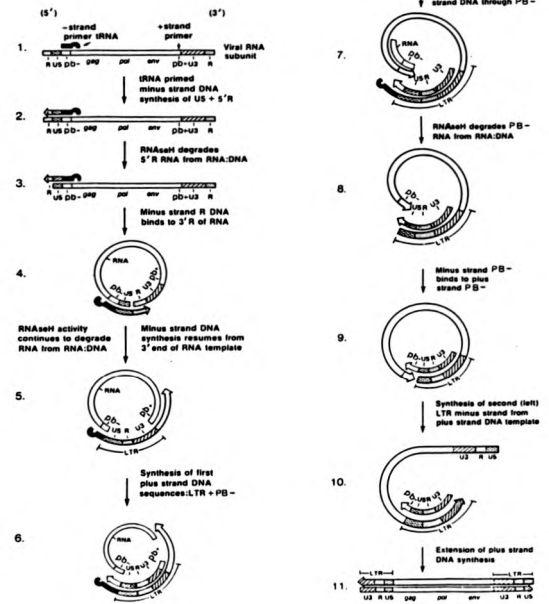

(5′)
(3′)
– strand primer tRNA
+ strand primer
1.
Viral RNA subunit
R US PB– gag pol env PB+U3 R
tRNA primed minus strand DNA synthesis of US + 5′R
2.
RNAseH degrades 5′R RNA from RNA:DNA
3.
Minus strand R DNA binds to 3′R of RNA
4.
RNA
RNAseH activity continues to degrade RNA from RNA:DNA
Minus strand DNA synthesis resumes from 3′ end of RNA template
5.
Synthesis of first plus strand DNA sequences:LTR + PB–
6.
Extension of minus strand DNA through PB–
7.
RNAseH degrades PB– RNA from RNA:DNA
8.
Minus strand PB– binds to plus strand PB–
9.
Synthesis of second (left) LTR minus strand from plus strand DNA template
U3 R US
10.
Extension of plus strand DNA synthesis
LTR
11.
U3 R US gag pol env U3 R US

Figure 6. Schematic representation of the formation of the Moloney-MuLV double stranded DNA provirus: reverse transcription

The replication strategy depicted is shown for simplicity for a single RNA subunit, although it is not clear whether one or two RNA subunits are involved in the process.

A detailed discussion is given in the appropriate text.

Adapted from Lowy (1985).

RNA molecule or to the second RNA subunit found within the virion. In both cases the minus strand synthesis, which contains only one R sequence can now be extended from the 3' end to the 5' end of the RNA (Figure 6.4). Preparations for the third step (synthesis of the minus strand 5' LTR) are made before the second step (elongation of the minus strand from the 3' end of the viral RNA) has been completed. The 3' LTR minus strand DNA is formed once the minus strand U3 sequences have been synthesized (Figure 6.5). At this stage the RNAse H presumably continues to degrade the RNA from the newly formed RNA:DNA hybrid sequences. Thus the plus strand LTR that serves as the template for the third step of minus strand synthesis can be synthesized (Figure 6.6, Gilboa et al.. 1979; Kung et al., 1981; Mitra et al., 1982). This plus strand DNA uses the minus strand LTR DNA as its template for the synthesis. Its synthesis apparently primed by virion RNA that remains just beyond the left hand boundary of U3 (PB+). Although this plus strand DNA is made from the 3' minus strand DNA it will eventually become the plus strand for the 5' LTR. This plus strand synthesis probably extends just beyond the LTR DNA sequences to make a copy of the first 18 nucleotides of the tRNA primer that is still linked to the minus strand DNA, since these sequences are apparently used in making the second template jump, described below (Taylor and Hsu, 1980). On the other strand, minus strand DNA synthesis from the RNA template is believed to stop after copying through PB(-) the 18 nucleotides that represent the tRNA binding site (Figure 6.7), since the RNA sequences (U5 and R) 5' to this site have been degraded by RNAse H thus the second stage is completed.

In the third step of minus strand DNA synthesis reverse transcriptase switches its template a second time, this time to the plus strand LTR.

This jump is apparently facilitated by the 18 nucleotides of this plus strand fragment that are complementary to the tRNA. The RNAse H activity then digests the tRNA from the viral RNA:DNA minus strand hybrid (Figure 6.8). These 18 nucleotides on the minus strand are then base paired with the complementary 18 nucleotides at the end of the plus strand DNA (Figure 6.9). The 5' minus strand LTR is then synthesized (Figure 6.10) thus completing this strand and displacing the 3' minus strand LTR from the plus strand LTR. Thus the plus strand LTR which was originally synthesized from the 3' LTR becomes the plus strand for the 5' LTR. The remainder of the plus strand is synthesized by 5'-3' extension, (Figure 6.11) thus generating the flush ended linear double stranded DNA with LTRs at both ends.

iv) Integration of the viral genome.

Once completed these linear molecules are transferred to the nucleus, where they are circularised. Circular DNA has only been recovered from the nuclear fraction of infected cells (Guntaka et al., 1976; Fritsch and Temin, 1977) and only after sufficient time has elapsed for linear DNA to be synthesized and transported to the nucleus (Shank and Varmus, 1978). These are conventionally described as circular molecules with two tandem LTRs or with one LTR, although various irregular forms which do not conform to this convention have been isolated by molecular cloning (Ju and Skalka, 1980; Shoemaker et al., 1981; Van Beveran et al., 1982). Clones with two complete LTRs in tandem are seldom found suggesting that they are rarely formed, or once formed are quickly integrated. The manner in which linear DNA is converted to the circular form remains conjectural, but impaired conversion in several experimental situations suggest that both viral and cellular factors

may have a role (Yang et al., 1980; Chinsky and Soeiro, 1982; Hubleitel and Aboud, 1983). Which of these forms is subsequently integrated was elegantly shown by Panganiban and Temin (1983), who used recombinant retroviruses to demonstrate that the circular form with two tandem LTRs is the immediate precursor to proviral DNA. The same workers also showed that the point of recombination leading to integration on the viral DNA is specific. Recombination always occurs at specific sites near the outer edge of the terminal repeat sequences (the so called inverted repeats IRs). For the murine viruses these sequences are 13 bp perfect inverted repeats present at the termini of the linear DNA. These sequences may be designated att sequences, analogous to those found in the bacteriophage lambda. When these termini are joined to form the circular DNA a perfect 26 bp (in the case of MuLV) palindrome is created which appears to act as a substrate for the integration reaction (Panganiban and Temin, 1983). The ability of these palindromic sequences (in ALV) to act as substrates for in vitro cleavage by the endonuclease activity (Duyke et al., 1983) also suggests that this structure is a precursor to the integrated provirus. However further analysis using Mo-MuLV in which one of the two termini of the LTR was altered showed that a perfect palindrome is indeed not essential and that the central four base pairs of the palindrome can be altered in sequence and in number without preventing integration (Colicelli and Goff, 1985).

The integration of the viral DNA into the host sequences has many analogies with integration by transposable elements in both prokaryotes and eukaryotes (Shoemaker et al., 1980; Shoemaker et al., 1981). Although the point of recombination on the viral DNA appears to be specific, the target sites on the host DNA are selected with no (or at

most low) apparent specificity at the sequence level, and there is no obvious pattern common to the target sites (Shimotohno and Temin, 1980). However it should be noted that there is some good evidence that the host sequences are not all equally good targets. Some host genes for example are "cold spots" for retroviral insertion (King et al., 1985) and transcriptionally active areas of the host genome may be "hot spots" for insertion, (Vijaya et al., 1986; Rhodewohld, PhD-Thesis, Hamburg, 1986; Rhodewohld et al., 1987). One consequence of integration of the viral DNA into the host cell genome, is that the two terminal nucleotides from each end of the free viral DNA are deleted. For the host DNA, proviral insertion results in duplication of 4-6 host nucleotides. One copy of these sequences (that were present once in the pre-integration site) now flank the left and right ends of the proviral DNA. Very little is known about the biochemistry of this reaction, although an in vitro system used to analyse this, will result in rapid progress (Goff and Lobel, 1987). The region known to be specifically required for the establishment of the integrated provirus, has been mapped to the 3' of the pol gene, the so called $p46^{pol}$ (integrase) (Schwartzberg et al., 1984; Dornhower and Varmus, 1984). One interesting aspect of this integration reaction is that integrated DNAs are themselves able to yield progeny virus, probably because the unintegrated DNAs are not exposed to the cellular synthetic machinery responsible for transcription, but are wrapped up in unaccessable structures. It has also been shown by studies in which naked DNA was transfected or injected into the cytoplasm of cells, that no specific integration occurs, which was interpreted to mean that virion proteins must remain associated with the viral nucleic acids after reverse transcriptase to afford successful integration (Luciw et al., 1984).

Integration is thus an essential step in the replication cycle and normally precedes transcription and the eventual formation of progeny. Biochemical studies followed the genetic (mutation) studies which defined the 3' portion of pol as coding for the p46^{pol} integrase. These show that this protein, although apparently cleaved from pr$^{gag-pol}$, is found in an active form within the virion closely associated with the reverse transcriptase (Hu et al., 1986). It had no endonuclease activity. Recently p46^{pol} has been expressed in bacteria and shown to be a potent DNA binding protein with preference for single stranded over double stranded DNA (Roth and Goff, pers. commun.). The enzyme retained its binding properties in 0.2 M salt and lower concentrations in the presence of EDTA (stringent DNA binding conditions) although no endonuclease activity has been associated with the purified enzyme.

v) Expression of the proviral DNA.

The integrated provirus represents a transcriptional unit that contains its own regulatory sequences. However unintegrated viral DNAs may also be transcribed, to give rise to viral progeny although very inefficiently (Cooper and Okenquist, 1978). Expression of a given provirus depends on host specific as well as viral specific factors which may include the site at which the provirus is integrated, the physiological state of the cell and the viral LTR. The viral gene products do not participate in the control of viral transcription per se although there are exceptions to this, notably the HIV related virus family (Sodroski et al., 1984; Chen et al., 1985; Felber et al., 1985). However in these systems the provirus depends entirely upon the host encoded synthetic machinery. As is true of most cellular genes the transcriptionally active proviruses are usually found in hypomethylated

regions of the DNA that are sensitive to DNAse I (Breatnach and Chambon, 1981; Groudine et al., 1981; Jaenisch et al., 1981; Breindl et al., 1982). In vitro methylation can prevent expression of cloned retroviral DNA genomes (McGeady et al., 1983; Simon et al., 1983). However unexpressed non-infectious proviruses after molecular cloning into bacteria, are not methylated and are infectious (Harbers et al., 1981). Conversely proviruses located at DNAse-resistant hypermethylated regions are usually unexpressed. Indeed integrated proviruses that are transcriptionally active have DNAse I hypersensitive sites within the 5' and 3' LTRs, sites which appear to correspond to the viral CAP and enhancer region (Thompson and Fan, 1985). Thus otherwise normal proviruses may be transcriptionally silent, presumably because of the influence of the flanking host sequences.

Virus specific regulation of expression depends principally on the LTR (Blair et al., 1980; Chang et al., 1980; Temin, 1981). These sequences contain signals for enhancement, promotion, initiation and polyadenylation of RNA synthesis and are depicted schematically in Figure 7. (Fuhrman et al., 1981; Gilmartin et al., 1983; Luciw et al., 1983; Wood et al., 1983). Various gene fusion studies have been performed on these LTRs to identify functions (Laimens et al., 1982; Levinson et al., 1982; Laimens et al., 1984; Schulz et al., 1985). Most retroviruses appear to have only one transcriptional initiation start site and one poly-A addition site which both lie within the LTR. The start site and the poly-A addition site define the R sequences found at both ends of the viral genome. The start sites coincide with the 5' end of both genomic RNA and the viral mRNAs (Cordell et al., 1978). Transcription of viral RNA begins at the end of the R sequences in the 5' LTR and as is true of most eukaryotic genes is catalysed by a

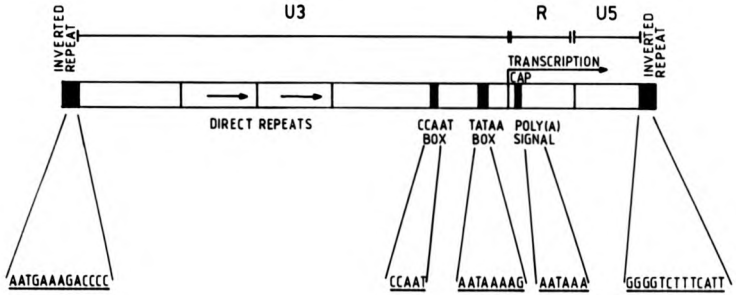

INVERTED REPEAT
U3
R
U5
INVERTED REPEAT
TRANSCRIPTION CAP
DIRECT REPEATS
CCAAT BOX
TATAA BOX
POLY(A) SIGNAL
AATGAAAGACCCC
CCAAT
AATAAAAG
AATAAA
GGGGTCTTTCATT

Figure 7. Organisation of control sequences in the Moloney-MuLV LTR

Assignment of control sequences to various regions of the proviral LTR. The inverted repeats (which form a palindromic sequence) are important for integration.
GGAAT and TATAA boxes are important for the correct initiation of transcription which starts at the CAP site in the 5' LTR and terminates at the poly(A) signal in the 3' LTR.
The direct repeats (74 bp) are not depicted here but contain the enhancer sequences (core sequence; TGTGGTAAG) responsible for elevated transcription levels.

cellular type II RNA polymerase (as shown by α-amanatin inhibition experiments, Dinowitz, 1975; Bishop et al., 1976). The LTRs of RSV and MMTV also function as good promoters in E.coli, although the transcriptional start sites used in bacterium are not the same as those used in eukaryotic cells (Mitsalis et al., 1981; Hermer et al., 1983; Prakash et al., 1983).

Each RNA transcript is polyadenylated post-transcriptionally near the 3' end of R in the 3' LTR a sequence of events that is initiated by a poly (A) signal (usually AATAAA) which is located in R approximately 20 nucleotides upstream from the right hand end of the R sequences. Thus the viral RNA transcriptions begin downstream from the left end of the provirus and their poly-A tract is added to sequences located upstream from the right end of the provirus.

The U3 regions of all LTRs also possess CCAAT (Efstratiadis et al., 1980) and TATAA (Corden et al., 1980) boxes or very similar sequences associated with RNA polymerase II promoter regions just upstream from the initiation site (Dhar et al., 1980; Shimotohno et al., 1980; Sutcliff et al., 1980; Yamamoto et al., 1980; van Beveren et al., 1980; Swanstrom et al., 1981). In the case of MSV a TATAA homology sequence is found 20-30 bp upstream of the putative start site of transcription (CAP site, van Beveran et al., 1981; Fuhrman et al., 1981), and a pentanucleotide sequence that is homologous to the CCAAT box consensus is located in the MSV LTR approximately 80 bp upstream of the mRNA CAP site. LTRs also contain sequences that enhance the efficiency of transcription in a position and orientation independent manner, being able to exert their effects over a long distance of 5 kb and more, (Banerji et al., 1981; Moreau et al., 1981; Fromm and Berg, 1982; Gluzman and Shenk, 1983; Khoury and Gruss, 1983; Weiher et al., 1983).

LTR enhancers, like enhancers in general, act entirely in cis and preferentially upon proximal promoters (de Villiers et al., 1983; Wasylyk et al., 1983). One other property of the enhancers is that they appear to display cell specificity as shown for the mouse immunoglobulin heavy-chain locus (where they have been found within the introns, Banerji et al., 1983; Gilis et al., 1983), and more recently for other systems (Gorski et al., 1986). Evidence that the retrovirus LTRs contain enhancers came originally from many sources, including the fact that the U3 region of the LTR could restore partial replication competence to SV40 DNA or augment the frequency of morphological transformation of mouse cells (Levinson et al., 1982; Kriegler and Botchan, 1983). Attention was drawn to the finding of 50-100 bp tandem repeats in the functional domain of the LTR because a similar (72 bp), but largely non-homologous duplication, forms the enhancer region of SV40. Although the duplication is found within many retroviruses its conservation does not appear to be essential, for there are a number of murine retroviruses that have only one copy or various combinations (see later in section on the Friend virus complex).

MuLV enhancer elements also appear to play an important role in the biological behaviour of these viruses, for the leukaemogenic potential of certain MuLVs has been assigned to a region in or near to the U3 region (Des Groseillers et al., 1983; Lenz and Haseltine, 1983; Lung et al., 1984; Lenz et al., 1984; Des Groseillers and Jolicoeur, 1984). Several results imply that a tissue responsive component of the LTR might determine pathogenicity by fostering efficient viral growth and expression in the target organ. Indeed Des Groseillers et al., (1983) have shown that the ability of a recombinant MuLV to infect thymocytes, the target cell for a T-cell leukaemia-inducing virus strain, is

conferred by the LTR and probably by a sequence within the U3 region. More in detail studies using the myeloproliferative sarcoma virus (MPSV; Kollek et al., 1984; Stacey et al., 1984) indicated that recombinants carrying all or only the U3 region of the Mo-MuSV LTR still transform fibroblasts in vitro but do not induce haemopoietic changes in mice (Stocking et al., 1985). To test whether the MPSV LTR was functionally different from that of Mo-MuLV from which it was derived, further recombinants were constructed replacing the MPSV LTR with that of Mo-MuLV. The resulting recombinant was at least two orders of magnitude less active than wild-type MPSV (Stocking et al., 1986). Further studies by Chatis et al., (1983) have demonstrated that the erythroleukaemic Friend-MuLV (F-MuLV) can be converted to a thymoleukaemic strain by a 621 nucleotide region, which includes the U3 region of Mo-MuLV, with the converse also being true (Chatis et al., 1984). These results have recently been substantiated by Holland et al. (1987). Evidence that the MuLV promoter or enhancer can behave in a cell-dependent manner in cell culture adds strength to the argument that the organotropism and oncogenicity of MuLV may be closely tied to the characteristics of the enhancer (Laimens et al., 1982; Spandidos and Wilkie, 1983; Linney et al., 1984).

Although the mechanism by which long range activation of promoter regions is achieved is not yet understood, several different lines of evidence have implicated the involvement of cellular factors that act in trans to potentiate promoter utilisation and that these so called transcription factors cause their specific effects by interacting with the specified DNA sequences. Most of the early work along these lines has been performed with various virus models with members of the papovavirus and the herpesvirus family and has recently been reviewed

by McKnight and Tjian (1986).

The involvement of cellular factors in enhancer activation has been demonstrated most clearly in the case of the glucocorticoid receptor. In an elegant series of experiments Yamamoto and colleagues showed that the LTR of MMTV behaves as an enhancer and that the hormone inducibility of MMTV transcription is mediated by direct binding of the glucocorticoid receptor protein to the MMTV enhancer (Chandler et al., 1983; Payvar et al., 1983). More recent studies have indeed shown that a variety of nuclear transcription factors from different cell sources bind to the enhancer region of MSV and Mo-MuLV (Johnson et al., 1987; Speck and Baltimore, 1987).

One interesting aspect of retroviral transcription is that it appears to be blocked to a great extent in either preimplantation embryos and pluripotent teratocarcinoma cells (Périés et al., 1977; Teich et al., 1977; Stewart et al., 1982). Efforts to understand the block to productive infection in teratocarcinoma cells have indicated that DNA methylation and probably other mechanisms are involved (Niwa et al., 1983; Gautsch and Wilson, 1983). On the other hand, Linney et al., (1984) have demonstrated that the Mo-MuLV promoter functions poorly in undifferentiated teratocarcinoma cells and if the enhancer domain of Mo-MuLV is replaced by the enhancer domain of a polyoma mutant are able to grow in such cells then the promoter strength is restored. Thus these results suggested that the initial block of retroviral gene expression in undifferentiated cells is due to the failure of the enhancer sequence to function in these cells. Indeed a recently isolated mutant of the myeloproliferative sarcoma virus (MPSV), PCMV which is expressed more efficiently than Mo-MuLV and MPSV (which is expressed more efficiently than Mo-MuLV in F9 cells) in teratocarcinoma

cells (F9 and PCC4) contains a mutation in the LTR (loss of one of the direct repeats in the enhancer) along with a further deletion in the gag region (Hilberg et al., 1987). Further analysis of this LTR has shown that the binding of transcription factors isolated from undifferentiated teratocarcinoma cells is different from the binding to wild type MPSV and also to Mo-MuLV. In this system it apears that a putative repressor protein found in undifferentiated PCC4 cells is unable to bind to the PCMV proviral LTR and thus is unable to suppress transcription from the PCMV LTR whereas the Mo-MuLV LTR is able to interact with it (M. Grez, pers. comm.).

vi) Processing of the viral RNA transcripts.

The coding regions of many eukaryotic genes are interrupted by stretches of non-coding DNA called introns. The introns are transcribed into precursor RNA and are subsequently removed in a process termed RNA splicing, (reviewed by Breatnach and Chambon, 1981; Breitbart et al., 1987). A central problem in understanding the mechanism of splicing has been the identification of the RNA sequences responsible for the accurate removal of introns. A large body of evidence indicates that the cis acting regions required for efficient removal of introns exert their function through the formation of a secondary structure which brings the 5' and 3' splice junctions in close proximity. Retroviral RNA also undergoes splicing (Figure 8).

Transcription of the genome which is initiated in the 5' LTR forms a long transcript spanning all three genes: the 3' end of the resulting RNA lies within the 3' LTR and is polyadenylated like any conventional message. The primary transcript is in one sense polycistronic, though not in the conventional prokaryotic sense. A portion of this RNA is

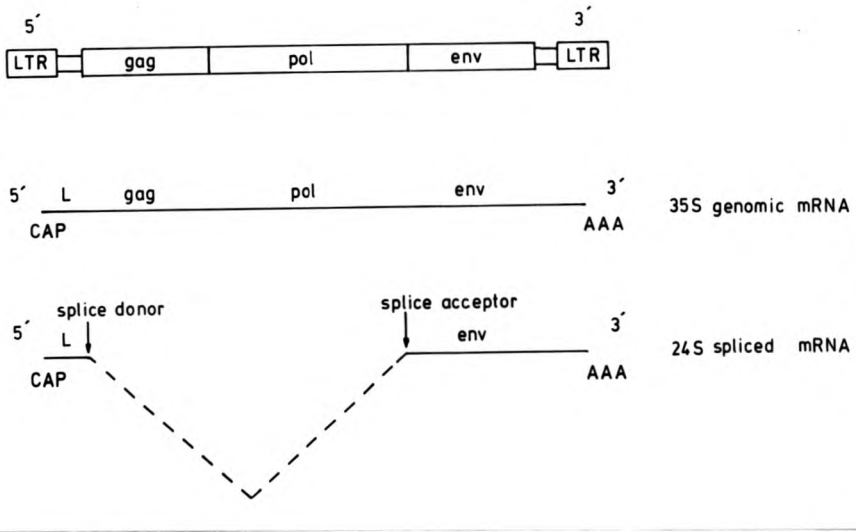

5´
3´
LTR
gag
pol
env
LTR
5´
L
gag
pol
env
3´
CAP
AAA
35S genomic mRNA
splice donor
splice acceptor
5´
L
env
3´
CAP
AAA
24S spliced mRNA

Figure 8. Transcription of the proviral DNA of Moloney-MuLV

Two transcripts have been positively identified and correspond to full length genomic RNA (35S) and a spliced version from which the *env* gene is translated. Both RNA species have 5' CAPs and 3' poly(A) tails as well as an untranslated leader sequence designated L.

The splice donor sequence for Moloney-MuLV (GAGGUA) is found approximately 320 bp upstream of the *gag* (p15) methionine (AUG) initiation codon and approximately 80 bp upstream from the start of the leader sequence.

The splice acceptor sequence (CUCUCCAAG) is found 280 bp upstream from the *env* AUG initiation codon, 70 bp before the end of *pol* ($p46^{gag\text{-}pol}$).

retained in intact form (35S) and another portion gives rise to a spliced derivative RNA consisting of an untranslated leader (L, about 200 nucleotides long) joined to *env* sequences (24S in the case of MuLV). It appears that the two RNAs are responsible for the formation of all the viral proteins (Fan and Baltimore, 1973; Gielkens *et al.*, 1974; Shanmugan *et al.*, 1974; van Zaane *et al.*, 1977; Fan and Verma, 1978; Rothenberg *et al.*, 1978). Although some investigators have detected smaller species of about 12-14S (Gielkins *et al.*, 1974).

One interesting feature of the *env* mRNA is that its acceptor site and some *env* coding sequences are located upstream from the *pol* termination codon; the *pol* and *env* gene open reading frames are in different phases (Schinnick *et al.*, 1981; van Beveren *et al.*, 1981; Seiki *et al.*, 1983; Schwartz *et al.*, 1983; Herr, 1984). The donor splice site for *env* mRNA is located upstream from *gag* in MuLV, but just downstream from the *gag* initiation codon in RSV. In RSV, *env* gene translation is initiated from the *gag* initiation AUG, whereas in MuLV the *env* gene translation is initiated from an AUG located within the portion of *env* that overlaps *pol* (Schwartz *et al.*, 1983).

No definitive structural differences between the viral genomic RNA subunit and the mRNAs that specify *gag* and *pol* proteins are known. However, their half-lives differ, suggesting that they are somehow assigned to different intracellular pools (Levin *et al.*, 1976).

vii) Viral protein synthesis and virion assembly.

Once the viral RNA has been expressed and modified appropriately, the virion proteins must be synthesized and assembled into particles that have encapsidated the viral RNA subunits (Dickson and Peters, 1982; Eisenman and Vogt, 1978; Oroszlan and Gilden, 1980; Stephenson, 1980).

The envelope of the virion is formed at the plasma membrane as the virus is released from the cell by a process called budding. The C-type viruses assemble their viral cores at or near the plasma membrane, whereas the B- and D-type viruses can apparently form some of their cores in the cytoplasm. A good deal is known about the synthesis, processing and modification of the individual viral proteins, however there is much less information on the mechanism by which virions are assembled.

Viral protein synthesis is quite similar among the different retroviruses, although the molecular weights of proteins with similar functions differ among the retroviruses. In the MuLVs most protein cleavage appears to take place after virion assembly, since rapid harvest virions still contain large amounts of uncleaved polyproteins (Bolognesi et al., 1978). This observation suggests that the particular order of the different proteins within each polyprotein may help to orientate the polyprotein appropriately within the virion (see Figure 3).

The gag proteins give rise to the core proteins excluding the reverse transcriptase. For MuLV, the gag precursor polyprotein is $Pr65^{gag}$ and is cleaved to four proteins whose order in $Pr65^{gag}$ is NH_2-p15-pp12-p30-p10-COOH. It appears that these cleavages are mediated by a viral encoded protease (p14, Crawford and Goff, 1985). The MuLV gag protein exists in glycosylated and non-glycosylated forms (Figure 9). The glycosylated forms are cleaved from $gPr80^{gag}$, which is synthesized from a different inframe initiation AUG codon located upstream from the initiation AUG for the nonglycosylated $Pr65^{gag}$. Only non-glycosylated gag proteins are found in ALV. Deletion mutants of MuLV that do not synthesize the glycosylated gag are still infectious, thus raising a

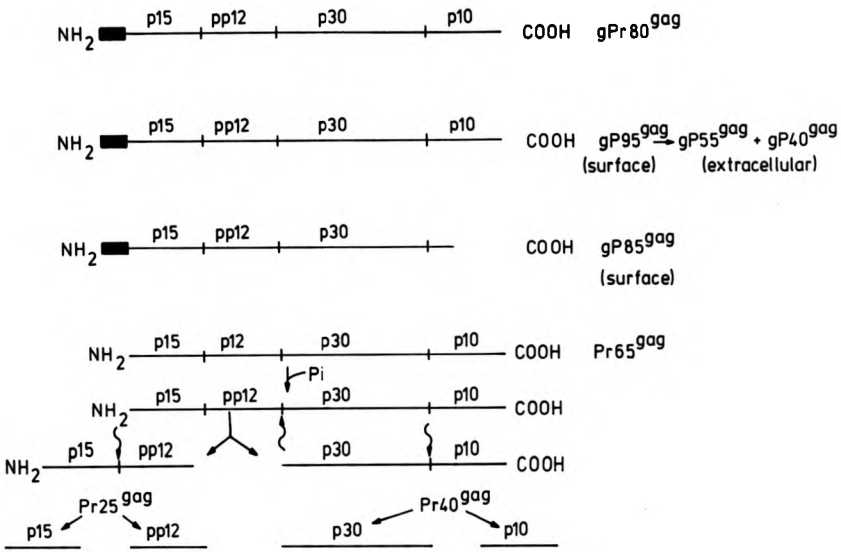

NH2
p15
pp12
p30
p10
COOH
gPr80gag
gP95gag → gP55gag + gP40gag
(surface)
(extracellular)
gP85gag
(surface)
p12
Pr65gag
Pi
Pr25gag
Pr40gag

Figure 9. Moloney-MuLV gag polyprotein synthesis

Depicted are the two metabolic pathways of glycosylated and non-glycosylated gag proteins.
Translation of both primary translation products occurs at different AUG codons, thus giving gPr80^{gag} an extra sequence at or near its NH_2 terminus (indicated by the shaded box). gPr80^{gag} is glycosylated at two positions in p15 and p30. gP95^{gag} is further modified by addition of extra oligosaccharides at the same positions.
Also shown is the phosphorylation of p12 to pp12 (phosphorylated form).


denotes cleavage sites.

question over the importance of the glycosylation events (Fan *et al*., 1983; Schwartzberg *et al*., 1983). Possibly they are necessary for *in vivo* infections. The p15^{gag} is very hydrophobic and in the virion it is probably located between the envelope and the capsid. Significant amounts of p15 copurify with the viral envelope (Stephenson, 1980) and the fatty acid, myristic acid is linked to it (Henderson *et al*., 1983). The hydrophobicity of p15 may enable Pr65^{gag} to align with the membrane during virion morphogenesis (Bolognesi *et al*., 1978). pp12 is an acidic protein that is phosphorylated at serine residues. Its precise location within the virion is not clear. It can bind to virion RNA, but it does not purify with the viral cores from which the envelope has been stripped *in vitro*. It has been proposed that most pp12 is located on the inner coat between the capsid and the envelope (Bolognesi *et al*., 1978). p30 is the major core protein and mutations within this region completely abolish virion assembly (Schwartzberg *et al*., 1984). It can self-assemble *in vitro*, which is consistent with its role as the principle capsid protein. Data concerning Fv-1 restriction would also suggest that p30 is possibly involved with integration of the proviral DNA. p10 is a highly basic protein that is bound to the virion RNA, forming the basis of ribonucleoprotein complex.

Many aspects of the *pol* gene products have been discussed earlier and will thus not be further mentioned here.

The *env* gene product Pr90^{env} is glycosylated and cleaved to gp70 and p15E, which remain bound to each other via a disulphide linkage. It is probable that p15E is a transmembrane protein with its C terminus located internal to the lipid membrane and its N terminal located external to the membrane (Herr, 1984; Pinter and Honner, 1983). In electron micrographs p15E probably represents the spikes on the viral

envelope while gp70 is the knob that surmounts the spike.

It is not known how the virion selectively encapsidates the two viral RNA subunits, or how the dimer RNA structure is formed. Sequences located between the donor splice site and the *gag* initiation AUG are required for packaging of the RNA into virions (Mann *et al.*, 1983; Watanabe and Temin, 1983). Deletion of sequences between the donor splice site and the initiation AUG of *gag* results in a mutant that synthesizes the viral proteins normally and can rescue replication defective viruses, but the deletion prevents it from packaging its own RNA into virions. The binding of tRNA to the viral RNA is apparently mediated in part by the reverse transcriptase which binds specifically to the appropriate tRNA (Panet *et al.*, 1975). However data from Goff's group would suggest that the stringency of the tRNA primer is not important for they were able to isolate a mutant Mo-MuLV which contained a new primer binding site and thus utilised a new tRNA (glutamine) to prime DNA synthesis (Colicelli and Goff, 1986). The formation of the dimer structure is probably mediated by the p10 protein since it has not been possible to form dimer structures from RNA subunit monomers. Retroviruses like most other viruses leave the host cell by a process of budding with the specific mechanism involved in this reaction being at the moment unclear. Recently a standardised and simplified nomenclature for proteins common to all retroviruses has been proposed (Leis *et al.*, 1988).

2. Haemopoiesis in the mouse.

Before describing the biology of the Friend virus complex a very brief account of haemopoiesis and erythropoiesis will be presented as a basis for the understanding of the disease caused by this virus

complex.

The formation of different types of blood cells is essential for normal development. Mature haemopoietic cells perform a variety of important functions, yet are relatively short lived and therefore require constant replacement. This requirement for constant replenishment has resulted in the haemopoietic system being divided into three main compartments: the mature end cells, a transit population of progenitor and differentiating cells, and a multipotential stem cell compartment that produces cells for the transit population (Figure 10). The pluripotential stem cell (CFSI) seen at the top of this scheme is able to repopulate a lethally irradiated mouse in both the myeloid and the lymphoid systems whereas the multipotential stem cell (CSFII) appears only able to repopulate cells within the myeloid system. Because of the large cell turnover required there is considerable amplification, particularly within the transit population in order to produce the required numbers of mature end cells. The rapid changes in mature haemopoietic cell numbers observed under particular conditions (i.e. changes in oxygen tension or infections), compared to the relatively constant numbers of such cells during steady state haemopoiesis, suggest constant and complex regulatory systems. Abnormalities in the normal development programme for blood cell formation result in various types of haematological disease.

a) Haempoietic progenitor cells.

Haemopoietic progenitor cells are the immediate progeny of multipotential cells and are primarily characterised by being committed to one, or at most two, haemopoietic lineages. The majority of

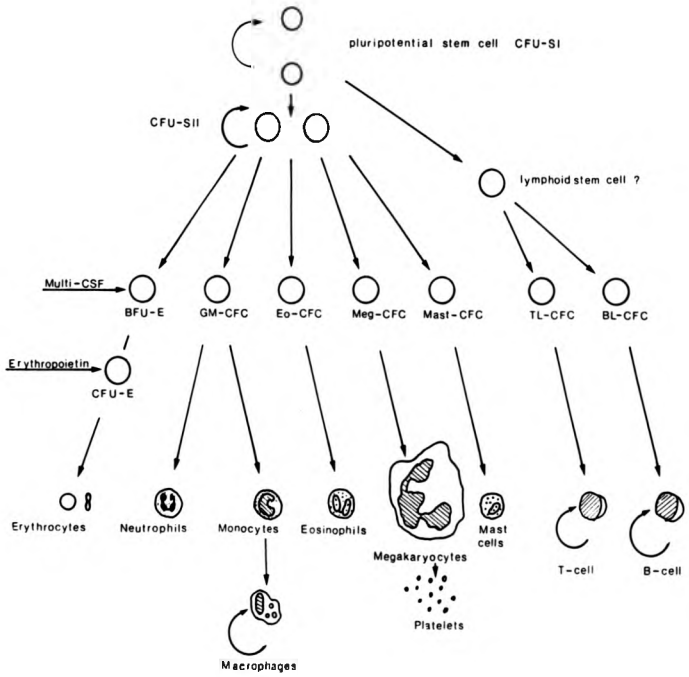

pluripotential stem cell CFU-SI
CFU-SII
lymphoid stem cell ?
Multi-CSF
BFU-E
GM-CFC
Eo-CFC
Meg-CFC
Mast-CFC
TL-CFC
BL-CFC
Erythropoietin
CFU-E
Erythrocytes
Neutrophils
Monocytes
Eosinophils
Megakaryocytes
Mast cells
T-cell
B-cell
Platelets
Macrophages

Figure 10. The pattern of differentiation in the murine lymphohaemopoietic system

Adapted from Ostertag et al. (1987). For discussion see text (reviewed by Ostertag et al., 1987).

The pluripotential stem cell, CFU-SI is only assayable in vivo, being the most primitive stem cell defined.

CFU-SII is defined as being multipotential, in that it has a limited repopulation capacity and is confined to the myeloid lineage. The other cells are defined below.

Erythroid progenitor cells;

BFU-E; burst forming unit-erythroid.

CFU-E; colony forming unit-erythroid.

Neutrophil-macrophage progenitor cells.

GM-CFC; granulocyte-macrophage colony-forming cell.

Eosinophil and megakaryocyte progenitor cells.

Eo-CFC; eosinophil colony-forming cell.

Meg-CFC; megakaryocyte colony-forming cell.

Mast progenitor cell.

Mast-CFC; mast colony forming cell.

Multi-CSF; multipotential colony stimulating factor is able to stimulate proliferation and differentiation of many of the cells seen in Figure 10. However it is only shown here for its stimulating activity within the erythroid pathway.

progenitor cells for the erythroid, eosinophil and megakaryocyte lineages appear to exist as unipotential cells, although some evidence has been presented to suggest that there may be minor subpopulations of bipotential/tripotential erythroid and megakaryocyte progenitors.

b) Erythropoiesis.

The cells which are capable of clonal differentiation within the erythroid compartment exist as heterogeneous populations, as can be seen in Figure 10. This heterogeneity is evident from the incubation period required in the in vitro colony assay system (methods reviewed by Till and McCulloch, 1980; Metcalf, 1984) for haemoglobin containing progeny to appear within a clone and by the number of cell divisions occuring prior to haemoglobinisation.

The relatively mature erythroid progenitor cells, termed CFU-E form colonies of haemoglobinised cells (of 80-200 cells) after a 48 h incubation period in vitro (Stephenson et al., 1971). After longer incubation periods (up to 21 days) larger multicentric haemoglobinised colonies appear in cultures, and these colonies are produced by erythroid progenitor cells termed BFU-E (Axelrad et al., 1974; Gregory, 1976). BFU-E may be further sub-divided according to the day of incubation at which they produce recognisable haemoglobinised progeny (Gregory and Eaves, 1978). The BFU-E which appear to be haemoglobinised after long incubation periods are considered to be the most primitive (these cells also form the most progeny of up to 20,000 cells in one colony). The available evidence would suggest that the more primitive BFU-E are direct progeny of multipotential stem cells, whereas the later BFU-E give rise directly to CFU-E. Analysis of enriched CFU-E fractions have shown that these cells are basophilic blast cells and

their immediate progeny are most likely proerythroblasts (Nicola et al., 1981).

Manipulation of the circulating levels of erythrocytes and subsequent studies of BFU-E and CFU-E numbers in the spleen and bone marrow has suggested that these two populations of cells are directly regulated. CFU-E are maximally stimulated by erythropoietin (epo) whereas epo (which is mainly produced by an as yet unknown cell type in the kidney, Bauer, 1986; Caro et al., 1986, and in some cases in the liver, Goldwasser et al., 1986, and recently in a well defined in vitro hepatocyte system, Goldberg et al., 1987) has no effect upon the primitive BFU-E differentiation and minor effects on the mature BFU-E although insulin like growth factor 1 (IGF1) can replace epo for maximal stimulation of CFU-E cells (Kurtz et al., 1982, 1985). In addition a subset of CFU-E may also be stimulated by a non-epo molecule (Iscove, 1978; Fagg, 1981).

BFU-E proliferation and differentiation is maximally stimulated by multi-CSF, with smaller subsets of multi-CSF responsive BFU-E being also responsive to GM-CSF and G-CSF (Metcalf et al., 1980; Metcalf and Nicola, 1983). This two tiered model of erythropoietic stimulation observed in vitro agrees with the in vivo data, since in those situations where circulating epo levels are reduced (i.e. hypertransfusion) CFU-E numbers decline whereas BFU-E numbers remain unaffected (Iscove, 1978).

3. The Friend virus complex.

a) Isolation and history.

Electron microscopic observations on Ehrlich ascites carcinoma cells which demonstrated the presence of virus like particles (Selby et al.,

1954) led C. Friend to attempt to isolate these virus like particles and assay them for biological activity. Cell-free filtrates prepared from these tumour cells were inoculated into newborn Swiss mice. After an incubation period of 14 months it was discovered that none of the mice had developed carcinomas. However a splenomegaly was observed in a few of the mice and subsequently the Friend virus complex was isolated from one of the spleens that was identified as being leukaemic upon histological examination (Friend, 1957).

b) Pathogenesis of the original virus isolate.

Filtrates of the leukaemic spleens inoculated into adult Swiss mice induced hepatosplenomegaly and profound anaemia after a short incubation period of 2-3 weeks. The early phase of the disease was characterised by erythroblastosis. All leukaemic mice died within 1-3 months (Friend, 1957). Examination of the bone marrow, spleen and the liver showed extensive infiltration with large mononuclear hyperbasophilic cells, with the lymph nodes and thymus not being involved. The white cell count (normally 7000-10,000/ml) usually remained under 50,000 in the first few weeks p.i. with an increase occuring between the 5th and 7th week p.i. and then finally reached a count of 300,000. As the white cell count rose the red cell count decreased markedly and nucleated red cells were present in the peripheral blood. The cells of leukaemic tissues from the mice in the terminal stages of the disease formed tumours upon subcutaneous implantation into syngeneic mice which were transplantable (Friend and Haddad, 1960). The erythroid nature of the tumour cells was revealed after propagation in vitro (Friend et al., 1966; Friend et al., 1971) and after the formation of spleen colonies in irradiated mice (Rossi

and Friend, 1967). These cells, predominantly proerythroblasts which exhibited erythroid maturation, as demonstrated by both morphologic and functional criteria such as ^{59}Fe uptake and synthesis of haemoglobin. Friend leukaemia cells which had been tissue culture adapted produced haemoglobin after induction with DMSO or other agents (Friend et al., 1971; Steinheider et al., 1971; Ostertag et al., 1972).

After passage of the original anaemia-inducing strain from C. Friend to other laboratories Mirand (1967) noticed that certain stocks of the Friend virus were able to induce marked erythropoiesis in susceptible (see genetic control of Friend virus infection) mouse strains suggesting the direct effect in vivo of Friend virus upon erythroid precursor cells.

Distribution of the original strain of Friend virus to a number of laboratories throughout the world led to the selection of distinct lines of Friend virus. For example Mirand noticed that certain preparations of Friend virus induced a marked polycythaemia (i.e. an increase in the number of immature erythroid cells in the blood) and not anaemia, in inoculated mice and referred to these virus preparations as polycythaemia inducing strains of Friend virus (FV-P) as opposed to the anaemia inducing strains (FV-A: Mirand, 1967; Mirand et al., 1968). Mirand observed that FV-P strains markedly stimulated erythropoiesis in hypertransfused polycythaemic mice, as determined by radioactive ^{59}Fe incorporation, whereas FV-A strains and Rauscher murine leukaemia virus were unable to induce such autonomous erythropoiesis (Mirand et al., 1971; Mirand, 1976).

A number of virus stocks which were obtained independently from Dr. Friend and have been passaged in different laboratories such as those of Axelrad (Axelrad and Steeves, 1964), Mirand (Mirand, 1968), Lilly

(Lilly, 1970) and Ikawa (Ikawa et al., 1976) were also found to induce polycythaemia following inoculation of susceptable mice. In spite of the differences in the haemopoietic changes induced by different preparations of the Friend virus, both the FV-P and FV-A strains exhibit several properties by which the Friend virus can be distinguished from previously studied murine leukaemia viruses:

1) A short latent interval.
2) Pathogenicity for adult as well as newborn mice.
3) Lack of thymic or lymph node involvement.
4) Predominant involvement of cells of the erythroid series in the leukaemic process.

c) Two viral components involved in the Friend disease.

From a variety of genetical and biological studies (by physical methods and by isolation of non-producer cell lines upon end point dilution) using FV-P it was suggested that most if not all Friend virus preparations contain at least two discrete viral entities (Fieldsteel et al., 1969; Steeves and Mirand, 1969; Bentvelzer et al., 1972; Steeves, 1975; Troxler et al., 1977; Pragnell et al., 1978; Bilello et al., 1980; Ruta and Kabat, 1980; Troxler et al., 1980). The two component nature of the FV complex was also established by analysis of the viral RNA (Maisel et al., 1973) with the RNA molecules coding for the helper virus and for SFFV functions, being associated as homodimers in the virions, of the FV complex, (Dube et al., 1976).

a) Replication defective, rapidly transforming virus which was designated spleen focus forming virus (SFFV) either SFFVp for the polycythaemia inducing strain or SFFVa for the anaemia inducing strain.

b) Replication competent type-C helper virus (F-MuLV) which was

necessary for the successful transmission and spread of SFFV. Thus the Friend virus is often referred to as the Friend virus complex.

d) Effects of SFFVp on haemopoietic cells.

Infection of susceptable mice with the SFFVp viral complex results in a rapid and massive proliferation in the spleen of hyperbasophilic proerythroblast like cells, Friend leukaemia cells (FLC, Tambourin and Wendling, 1971). If low doses of the virus are used then one can see individual macroscopic spleen foci after 9-16 days p.i. an observation which led Axelrad and Steeves (1964) to use this criteria as a titration system for SFFV (also Mirand et al., 1968). The spleen and liver become greatly enlarged by three weeks p.i. and due to active erythropoiesis, a polycythaemia develops (Mirand et al., 1961; Tambourin and Wendling, 1971). The disease proves fatal in 3-6 weeks.

Erythropoiesis is greatly affected in that the erythroid differentiation which is normally under the control of epo is seemingly uncontrolled. The infected mice continue to produce mature red cells under conditions where epo levels are undetectable and erythropoiesis should thus cease (Mirand, 1967; Mirand et al., 1968; Sassa et al., 1968; Tambourin and Wendling, 1971). When haemopoietic cells from these mice are tested in the in vitro CFU-E assay then one sees a large increase in the number of these colonies (mostly in the spleen but also, depending on the strain, in the bone marrow) beginning at approximately 3 days p.i. (Horoszwicz et al., 1975; Liao and Axelrad, 1975; Hankins and Krantz, 1975; Fagg et al., 1980; Fagg and Ostertag, 1982). While normal cells require epo for CFU-E formation, cells from SFFVp infected mice are able to form CFU-E in the absence of an

exogenous epo source. Further in vitro studies have shown that infection of bone marrow cells with SFFVp that had been pseudotyped with any of a number of MuLVs and then grown in semi-solid media in the absence of epo resulted in the growth of large erythroid clusters (bursts) after approximately 7-10 days (Hankins et al., 1978). These bursts are the progenitor cells of the CFU-E, the BFU-E. These virally induced BFU-E (vBFU-E) are again epo independent. From such in vitro studies it has also been shown that the pool size and self renewel capacity of pluripotential haemopoietic stem cells increases after continuous culture of bone marrow cells with SFFV (Eckner et al., 1982; Greenberger et al., 1983) although haemopoietic cell line isolated from such stem cell cultures have been shown only to contain F-MuLV and no SFFV (Kluge et al., 1986).

Although SFFVp is able to infect a variety of haemopoietic cells (and also expresses in these cells, Colletta et al., 1983) it appears from a variety of studies that the target cell is indeed an epo responsive erythroid cell (Tambourin and Wendling, 1971; Opitz and Seidel, 1978; Kost et al., 1979, 1981). In the studies by Kost and co-workers it was suggested that the target cell is a relatively late committed erythroid precursor cell that is located between the day 8 BFU-E and the day 2 CFU-E. In these studies it was also shown that the target cell for SFFV must be in a state of active cellular DNA synthesis in order for the virus to have its effect. However the exact target cell of SFFVp has not been conclusively shown even in studies using sorted and enriched erythroid precursor cells for SFFVp infection in vitro (Johnson and Ostertag, pers. commun.). However such studies have not been easy to do since until recently no biologically active SFFVp virus containing a selectable marker gene has been available to monitor the infection

process (Hunt et al., 1987, and unpublished data).

e) Effects of SFFVa on haemopoietic cells.

SFFVa infected mice in contrast to SFFVp infected mice do not become polycythaemic but develop a mild anaemia (Steinheider et al., 1979). Unlike in SFFVp infected mice, erythropoiesis in SFFVa infected mice is sensitive to the normal physiological controls in that the CFU-Es (although increased in number) are not able to form in vitro without an exogenously added epo source (Tambourin et al., 1979; Steinheider et al., 1979; MacDonald et al., 1980). Interestingly SFFVa infected erythroid cells show an increase (two orders of magnitude) in the number of epo receptors on their cell surface (Krantz et al., 1987). As with SFFVp, infection of normal bone marrow cells in vitro with SFFVa also leads to the development of vBFU-E (i.e. those BFU-Es seen after viral infection and thus induced by the infection, Hankins and Troxler, 1980; Koury et al., 1982). However such bursts do not differentiate into mature erythroid cells unless a small amount of epo is added.

Although SFFVa appears to interfere less than SFFVp with erythropoiesis both viruses increase the proliferative capacity of erythroid precursor cells and enable them to differentiate under conditions that are suboptimal for uninfected cells. The target cell for SFFVa is thus probably very close to or identical to that for SFFVp.

f) Pathogenesis of the helper virus.

Steeves and Lilly (1971) and others (reviewed by Tambourin, 1979; Troxler et al., 1980; Ostertag and Pragnell, 1981) have shown that the ecotropic F-MuLV is able to induce a splenic leukaemia after

inoculation of susceptible newborn mice (Troxler and Scolnick, 1978; MacDonald et al., 1980; Oliff et al., 1980; Troxler et al., 1980; Ishimoto et al., 1981; Ruscetti et al., 1981). Two types of F-MuLV have been closely analysed after biological cloning: One has been termed the Friend lymphatic leukaemia virus (F-LLV, Fieldsteel et al., 1969) which induces a lymphatic leukaemia 2-4 months p.i. of newborn animals. The second type F-MuLV, induces an erythroid hyperplasia after a somewhat shorter latency period after inoculation of newborn mice (6-9 weeks, compared to that induced by other ecotropic helper viruses, such as Mo-MuLV (Troxler et al., 1980). F-MuLV induces erythroleukaemia associated with a rapid increase in BFU-E (Niho et al., 1982). Permanent erythroid lines isolated from such mice can be induced to differentiate in vitro (Oliff et al., 1981), some of which may be blocked earlier in differentiation than F-SFFV transformed cell lines (Shibuya and Mak, 1983a). In accordance with the slower onset of disease as compared to that induced by SFFV, infection of newborn mice with MuLV generated a new recombinant virus, the mink cell focus-inducing virus (MCFV). This virus is characterised by its dual tropism (Hartley et al., 1977). Friend MCFV (F-MCFV) causes an accelerated erythroid hyperplasia (compared to F-MuLV) when injected into newborn mice (Troxler et al., 1978). The induction of erythroblastosis was analysed in detail by using different mouse strains inoculated with F-MuLV. MCFV appears to interfere with helper virus-induced disease, since mice which express endogenous MCFV env proteins (C57BL/6 and DBA/2) are remarkably resistant to F-MuLV induced disease (Ruscetti et al., 1981, 1985). Indeed a strong correlation between cellular resistance to F-MuLV infection and expression of a glycoprotein has been reported (Kai et al., 1986).

Inoculation of adult rats or mice with F-MuLV results in the development of a lymphoid leukaemia after a latent interval of up to 6 months.

F-MuLV may be assayed in vitro by either of two assays:

a) The reverse transcriptase assay.

b) The XC plaque assay, both of which are described in the Materials and Methods section.

The disease specificity of the helper viruses appears to be determined by the viral LTR (Bösze et al., 1986), since recombinants between F-MuLV and Mo-MuLV as well as between F-MuLV and other MuLVs constructed by exchanging fragments containing the 3' end of the genomes demonstrate that the LTR is responsible for the tropism of the viruses (Chatis et al., 1983, 1984; Oliff et al., 1984; Holland et al., 1985).

In a more recent paper is has indeed been demonstrated by the use of a retroviral vector that the LTR of F-MuLV, or to be more precise the enhancer, determines erythroid specific gene expression (Holland et al., 1987).

g) Transformation of cells in vivo by Friend virus.

While it is possible to establish transplantable cell lines from diseased tissues of SFFV-infected mice late in the course of the disease the majority of the proliferating cells early in the diseased animal have a limited self renewal capacity and fail to grow in either syngeneic donor mice or as established cell lines in vitro. It has thus been postulated that the disease induced by SFFV has multiple stages.

i) An early stage in which the accumulating erythroid precursor cells have a finite life span but the size of the compartment of erythroid

precursor cells is uncontrolled.

ii) A late stage (after three weeks p.i.) which represents the proliferation of truly malignant cells that are capable of autonomous growth and extensive proliferation and tumourigenic growth in vivo (Tambourin et al., 1979, 1980; Levy et al., 1979).

It is not known whether the cells proliferating in the first stage are the progenitors of the malignant cells in the latter stage or whether the two populations of cells arise independently. Most of the cell lines derived from the malignant stage are more or less blocked in their ability to differentiate although they can be induced to differentiate with certain chemicals such as DMSO (Friend et al., 1971). Both SFFV and helper viral proteins can be detected at all stages of the disease, although several groups have shown in a variety of Friend leukaemia cells (FLC) alterations in expression (Ruscetti and Scolnick, 1983; Shen et al., 1983; Rovinski et al., 1987) and structure of the p53 locus (Rovinski et al., 1987) an oncogene thought to be involved in the cell cycle. This could possibly be due to integration of SFFV near to one of the alleles, thus leading to dysfunction of p53, which is thought to be important in the control of the cell cycle (Rovinski et al., 1987). However a second fraction of FLC are not altered in the expression of p53. Thus the question remains whether p53 activation is the primary event in causing malignancy or just a consequence of later mutational changes in the course of malignant progression. Very recent data presented by Tambourin's group (Moreau-Gachelin et al., 1988) have shown that in 95% of the tumours induced by SFFVp then this virus (although the helper is also present) is integrated adjacent to a putative oncogene Spi-1 thus activating this gene (or increasing its expression) by insertional mutagenesis.

b) Genetic control of Friend virus infection.

Genetic loci have been described that interfere with the rapid induction of erythroblastosis in mice by the Friend virus complex, Fv-1 and Fv-2. The Fv-1 locus can selectively inhibit infection by and replication of particular MuLVs as described earlier. Other loci that determine resistance to helper virus infection and/or replication also include the Fv-4 locus (Odaka et al., 1983; Yoshikura and Odaka, 1982; Ikeda et al., 1985) and also possibly the Rmcf^r locus (Ruscetti et al., 1985).

The Fv-2 locus, as first described by Odaka's group (Odaka and Yamamoto, 1962; Odaka, 1969; Odaka, 1973; Lilly and Pincus, 1973; Steeves, 1975), has two allelic forms, Fv-2^s and Fv-2^r (for absolute sensitivity and absolute resistance, respectively to in vivo induction of spleen foci and erythroleukaemia by SFFV, Odaka, 1970; Lilly and Pincus, 1973). The prototype Fv-2 resistant mouse strain is C57Bl/6 (Fv-2^{rr}); mice of most other inbred strains (e.g. BALB/c, DBA/2) are Fv-2^{ss}. Fv-2^s is the dominant allelic form, so that mice that are genetically heterozygous at this locus (Fv-2^s/Fv-2^r) are completely sensitive to Friend viral erythroleukaemogenesis (Odaka, 1969; Lilly, 1970). Fv-2 differs from Fv-1 in several aspects.

a) Unlike Fv-1 restriction, which can be overcome with increasing m.o.i., resistance or susceptibility conferred by the Fv-2 locus is absolute.

b) Susceptibility (Fv-2^s) is dominant over resistance (Fv-2^r) at the Fv-2 locus, in contrast to the effect of the Fv-1 gene.

c) Whereas Fv-1 restriction is active in murine fibroblast cells in culture, SFFV is able to replicate well in murine fibroblasts of Fv-2^r mice (Eckner and Hettrick, 1977; Evans et al., 1980). The action of

Fv-2^r seems restricted to haemopoietic or even possibly erythroid cells. Other loci which determine resistance to SFFVs include W/W^v and Sl/Sl^d (only for the SFFVp/F-MuLV complex; Russell, 1979), the mpsv resistance locus (specific for MPSV and R-SFFV/MuLV; Ostertag et al., 1981; Hess et al., 1984) and the putative Fv-5 locus determining specificity to the Friend virus complex (Shibuya and Mak, 1982a,b; Kitagava et al., 1983).

i) Genome organisation of F-MuLV and SFFV.

The genome organisation of F-MuLV will not be described here because of its similarity to that of Mo-MuLV (except for various point mutations and several recombinations in the env gene) which has been described earlier. However the genome organisation of F-SFFV because of unique structure will be described in detail.

After the SFFVp had been biologically cloned free from its helper virus (Troxler et al., 1977; Bernstein et al., 1977) its genome was compared with that of its natural ecotropic helper F-MuLV. Such studies have been carried out on several different strains of SFFV, all which had been derived from the original Friend virus stock and it appears that SFFV arose by recombination between F-MuLV and endogenous mouse genetic sequences (Troxler et al., 1977 a,b). These latter sequences are closely related to the env genes present in MCF viruses which have also arisen by a similar manner (Famulari, 1983).

Consistent with their replication defectiveness, both SFFVa and SFFVp contain a smaller genomic RNA (depending on the strain analysed, 32S RNA; Maisel et al., 1973; Dube et al., 1976; Evans et al., 1979) than F-MuLV (38S) and sequences encoding a functional RNA dependent DNA polymerase are deleted (Bernstein et al., 1977). SFFV RNA contains

sequences related to the 5' end of the gag gene of F-MuLV (Bernstein et al., 1977; Barbacid et al., 1978; Ruscetti et al., 1980), however this appears to vary from strain to strain, resulting in the production of a 15,000-45,000 dalton protein as compared with the 65,000 dalton prototype precursor gag protein. It also contains env gene related sequences that encode a 52,000-55,000 dalton envelope glycoprotein (Racevskis and Koch, 1977, 1978; Ikawa et al., 1978; Ruscetti et al., 1978, 1979; Dresler et al., 1979) that is immunologically related to the gp70 of MCF viruses (Ruscetti et al., 1978, 1979). A 21S subgenomic spliced RNA codes for this SFFV envelope protein (Bilello et al., 1980; Ruscetti et al., 1980; Yoshida and Yoshikura, 1980). The env gene of SFFVp also contains information that results in the inefficient processing of the primary env gene product to a higher molecular weight form which appears on the cell surface (Ruscetti et al., 1979, 1980; Ruta and Kabat, 1980). Thus Friend SFFVp appears to have arisen by deletion and recombination between F-MuLV and envelope genetic sequences endogenously present in the mouse.

The 30S genome of SFFVa is slightly smaller than that of SFFVp but like SFFVp it also contains sequences that are closely related to the envelope gene of MCF viruses (Evans et al., 1980; Troxler et al., 1980). SFFVa encodes a 52,000-55,000 dalton envelope glycoprotein and a 45,000 dalton gag related protein (Troxler et al., 1980; MacDonald et al., 1980a). The level of membrane bound gp55 in SFFVa is below detectable levels (Ruscetti et al., 1981; Wolff et al., 1985).

Like other retroviruses the integrated SFFV genome contains two LTRs, 514 bp each, at either end (Clark and Mak, 1982). These sequences contain various signals involved in transcriptional control of the provirus as described earlier. However one striking difference in the

U3 region of SFFV compared to ecotropic MuLV is the deletion of one copy of the direct tandem repeat containing an enhancer sequence (Bestwick et al., 1984; Clark and Mak, 1984; Wolff et al., 1985). Several of the MCFs have either the same or different deletion also in this region (Adachi et al., 1984; Koch et al., 1984; Vogt et al., 1985) thus suggesting that those with identical deletions may well be the putative precursors to SFFV. Comparison of several SFFV LTR sequences reveals only very few variations. In fact, the differences between the LTRs of all SFFVs (about 95% homology, Bestwick et al., 1984; Wolff et al., 1985) are most likely random.

Unlike other defective acute leukaemia inducing retroviruses no unique cellular sequences have been found within the SFFV genome although SFFV do contain sequences that may be detected in the mouse genome (Chattopadhyay et al., 1982). These are viral *env* sequences related to those that have been acquired by MCF viruses. Using molecularly cloned SFFVp (of which four strains have been cloned, and range in size between 6.0 and 6.6 kb: Linemeyer et al., 1980; Yamamoto et al., 1981; Amanuma et al., 1983; Hunt and Ostertag, unpublished data), various studies have been able to determine which sequences are responsible for the pathogenesis of SFFV.

Heterogeneity of the genome size of different SFFVp is mainly due to deletion within the *gag-pol* region of the genome. Restriction map comparisons are presented later in the results.

j) Studies using genetically engineered mutants.

Studies by Linemeyer et al. (1981) demonstrated that a 2.4 kb subgenomic fragment from SFFVp was able, in a two stage transfection assay using either F-MuLV, Mo-MuLV or an amphotrophic helper virus, to

retain its biological activity. This fragment contained 2.0 kb from the 3' end (*env*) the LTR and 400 bp of non-coding 5' sequences. Studies using specific fragments and mutants showed that biological activity was coincident with gp52 expression (Linemeyer *et al*., 1982). Similar studies using molecularly cloned SFFVa showed also that the biological activity was confined to the *env* region (Kaminchik *et al*., 1982). Spontaneous mutants of SFFVp isolated by Ruta *et al*. (1983) had mutations within the *env* gene which inhibited their correct processing and transport to the plasma membrane and were also non-leukaemogenic, as was also found for analogous mutants of Rauscher-SFFV (Machida *et al*., 1984).

k) Similarity of the SFFV and MCF envelope genes.

Since the envelope genes of both Friend and Rauscher SFFVs appear to be crucial for the pathogenicity of these viruses, it is important to understand the structure of their *env* genes and gene products. The location of the MCF related sequences within the envelope region of SFFV was determined by comparisons of SFFV with MCF viruses at both the genetic and the protein level. Indeed an antiserum capable of precipitating MCF, but not ecotropic gp70 was able to precipitate a glycoprotein of 52,000-55,000 daltons from fibroblasts non-productively infected with Friend-SFFVp (Ruscetti *et al*., 1979), Friend SFFVa (Troxler *et al*., 1980), and Rauscher SFFV (Ruta and Kabat, 1980). Cas-SFFV, derived from wild mouse ecotropic virus was also shown to encode an MCF gp70 related protein of 50,000 daltons (Langdon *et al*., 1983).

Localisation of the MCF gp70 related region within the SFFV gp55

molecule itself was accomplished through the use of monoclonal antibody to gp55 in conjunction with partial protease digestion. Both Friend MCF gp70 and gp55 contain a 23K V8 protease fragment that is precipitated by a monoclonal antibody to SFFV gp55 (Wolff et al., 1984). The 23K fragment in Friend MCF gp70 was localised to the N-terminus (Wolff et al., 1982), suggesting that the MCF related region of gp55 was also at the N-terminus.

Published nucleotide sequences of the envelope genes of several SFFVs (Amanuma et al., 1983; Wolff et al., 1983; Clark and Mak, 1983) have made it possible to make detailed comparisons of these with a number of MCF envelope genes. For three isolates of the polycythaemia inducing variants of SFFV it has been found that the MCF related region of the SFFV env gene codes for more than two thirds of the gp55 molecule. This includes the leader peptide and amino terminus of the glycoprotein itself. For all of the isolates, the amino acid homology with Mo-MCF (Bosselman et al., 1982), AKR MCF 247 (Holland et al., 1983), or Friend MCF (Koch et al., 1984) gp70 in the analogous amino terminal region is greater than 90%. Interestingly there is a 67 amino acid region (starting at amino acid residue number 60 from the amino terminus) which is similar for all of these SFFV and MCF viruses (Figure 11).

1) The SFFV env gene product.

There are several features of the SFFV env gene product gp55 which are unique only to this retroviral protein. These features are obvious when gp55 is compared with the env proteins of other retroviruses (reviewed by Weiss et al., 1982). The primary translational product of the prototype envelope gene is a polyprotein containing gp70 and Pr15E (see Figure 12a). Due to a large deletion (approximately 600 bp) in the

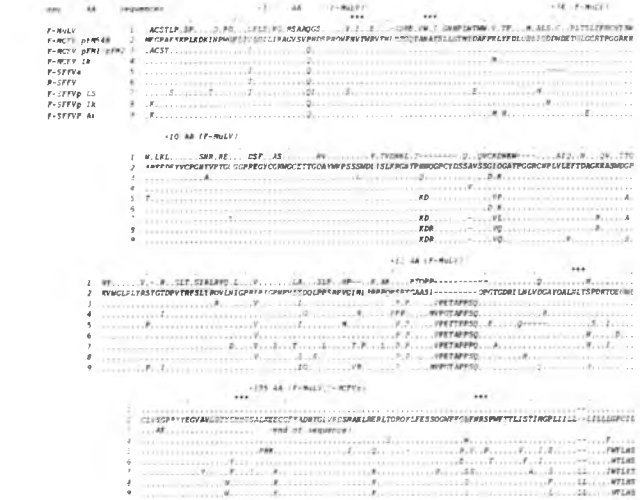


Figure 11. Amino acid sequence of SFFV and other related env proteins

env amino acid sequences of all the viruses depicted, were deduced from the nucleotide sequences and compared to the env amino acid sequence of F-MCF virus using the standard single letter code.

Symbol code.

-; missing residues.

***; possible common glycosylation sites.

For alignment and comparison part of the sequence was omitted (/). Recombination between xenotropic and ecotropic sequences are enclosed within the horizontal boxes (amino acid 270-300).

The sequence data are taken from:

Koch et al., 1984: F-MuLV, pF-MuLV57;

Koch et al., 1984: F-MCFV, pFMS4B and pFM1/pFM2;

Adachi et al., 1984: F-MCFV, FrNx;

Wolff et al., 1985: F-SFFVa;

Bestwick et al., 1984: R-SFFV;

Wolff et al., 1983: F-SFFVpLS;

Amanuma et al., 1983: F-SFFVpIK, K-1;

Clark and Mak, 1983: F-SFFV Ax;

Vogt et al., 1985: R-MCFV-1;

Bosselman et al., 1982: M-MCFV, c11b.

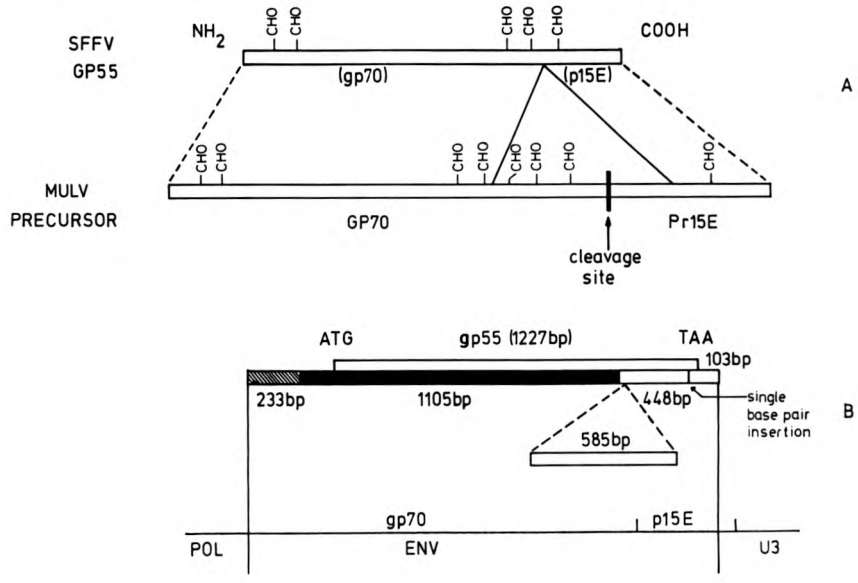

SFFV
GP55
NH2
CHO
COOH
(gp70)
(p15E)
A
MULV
PRECURSOR
GP70
Pr15E
cleavage
site
ATG
gp55 (1227bp)
TAA
103bp
233bp
1105bp
448bp
single
base pair
insertion
B
585bp
gp70
p15E
POL
ENV
U3

Figure 12. The SFFV env gene

a) Comparison of SFFV gp55 with the MuLV precursor *env* protein Pr90^{env}, to demonstrate the deletion of the gp70-p15E cleavage site.
CHO represents glycosylation sites.

b) Structure of the gp55 *env* gene as based on the nucleotide sequence of a molecularly cloned SFFVp strain (Wolff *et al*., 1983).
Open areas represent regions that are homologous to F-MuLV. The shaded area is non-homologous to F-MuLV but highly related to xenotropic and MCF viruses. The hatched area represents sequences where no nucleotide sequence relationship to F-MuLV was seen.

middle of the SFFV envelope gene which includes the cleavage site between gp70 and Pr15E, the amino terminal gp70 and carboxy terminal p15E sequences are joined and remain covalently linked (Figure 12b). This deletion was only discovered after sequencing the genomes of these SFFVs although heteroduplex analysis of SFFV and F-MuLV had previously indicated that a large deletion existed in the envelope gene of SFFV (Bosselman et al., 1980). However it was thought that SFFV was simply a truncated gp70 since it was not possible to precipitate gp55 with an antiserum prepared against p15E (Schultz et al., 1980). Evidence supporting the sequence data has come from tryptic peptide analysis of gp55 and p15E from both F-MuLV and Friend MCF (Wollf et al., 1984). The carboxy terminal domain of p15E which is translated as part of gp55 contains the transmembrane sequence of non-polar amino acids (Lenz et al., 1982; Pinter and Honnen, 1983; Koch et al., 1983) and presumably also provides a transmembrane anchor for the SFFV envelope glycoprotein, which is associated with membranes and is not secreted from the cell (Ruscetti et al., 1979; Dresler et al., 1979; Lyles and McConnell, 1981; Srinivas and Compans, 1983). This view is supported by the labelling of the membrane form of gp55 with ^{3}H palmitic acid (Srinivas and Compans, 1983) as this and other fatty acids are incorporated post-translationally into a wide variety of membrane glycoproteins and may serve to increase the affinity of glycoproteins for membranes (Schmidt and Schlesinger, 1980; Schmidt, 1982). The p15E related domain of gp55 is different from the prototype p15E in that its C-terminus is shorter and has a unique set of amino acids. This is caused by a single base pair insertion that shifts the reading frame. Such an insertion has been found in every SFFV *env* gene sequenced to date, including the *env* genes of three SFFVp and also SFFVa and

Rauscher SFFV (Bestwick et al., 1984; Hess et al., 1984). Interestingly neither the exact position of the insertion nor the particular base inserted is identical for each isolate, although the effects of the insertion in every case are to shorten the reading frame by 99 base pairs (33 amino acids) and produce a unique five to six amino acid sequence. Since all of these isolates have been passaged independently for many years it appears that these alterations must be critical for the generation of pathogenic SFFV.

The glycosylation pattern of gp55 is unique in that it has fewer glycosylation attachment sites than other env proteins. Sugar attachment at these sites is for the most part incomplete and heterogenous. Studies by several investigators have shown that the predominant form of gp55 that accumulates intracellularly contains immature oligosaccharides of the mannose type (Ruscetti et al., 1979; Dresler et al., 1979). This form of the protein has the same glycopeptide profile as the envelope precursors of F-MuLV and Friend MCF virus (Kemp et al., 1981) consisting of two major size classes of 2200 and 1500 daltons. Gp55 differs from these envelope precursors in the number of oligosaccharide attachment sites.

Although the majority of the gp55 accumulates intracellularly, as if unable to enter the golgi apparatus where the final sugar processing occurs, a small proportion passes through the entire pathway used for the processing of glycoproteins. The processed protein which incorporates a variety of complex sugars such as galactose, fucose and glucosamine has a molecular weight of 65,000 and can be detected on the cell surface (Ruscetti et al., 1979; Gillis et al., 1979; Ruta and Kabat, 1980).

The 65K SFFV envelope glycosylation pattern resembles more closely

the related Friend MCF gp70 than F-MuLV gp70 (Srinivas and Compans, 1983).

m) Molecular comparison of SFFVp and SFFVa.

Both SFFVp and SFFVa encode 52,000-55,000 dalton MCF gp70 related env proteins, which when analysed by tryptic fingerprinting, showed various differences (MacDonald et al., 1980a). These differences were also seen after oligonucleotide fingerprints of SFFVp and SFFVa RNAs (Evans et al., 1980). From these studies it could be seen that SFFVa specific oligonucleotides (as compared to SFFVp, F-MuLV and MCF viruses) were related at the 5' end of the genome, at a region 3' to pol and 5' to env. Further analysis using recombinant virus, constructed between molecularly cloned SFFVp and SFFVa strains have defined the env gene as being responsible for the disease caused by both SFFVp and SFFVa (Kaminchik et al., 1982).

At the nucleotide sequence level, all SFFVp have a 6 bp duplication at the 3' end of the env gene. This feature is not shared by R-SFFV and SFFVa, both of which induce anaemia, and do not confer complete independence to erythropoietin interaction of infected erythroid precursor cells.

There are a series of other shared features of the env region of the SFFVp which distinguishes them from SFFVa and which may have functional consequences.

a) All SFFVp have the same mutations in position 328 and 348 which lead to an altered change (glutamine to lysine) in the env SFFVp.

b) The above mentioned duplication of two codons in SFFVp leads to an insertion of two leucine residues in position 388/389 in the hydrophobic domain of the env protein of SFFVp.

c) A serine residue in position 367 replaces a leucine residue in SFFVp. This replacement could alter the attachment of fatty acids, which may alter the membrane of the env protein of SFFVp as compared to that of SFFVa or R-SFFV.

Construction of a recombinant virus between SFFVp and SFFVa has clearly demonstrated that the 3' end of the env gp52 of SFFVp determines the biological differences (Ruscetti and Wolff, 1985). However these studies did not demonstrate whether sequences in either the gag or pol region do in fact play a minor role in the pathogenesis of SFFV as indeed has been shown for F-MuLV.

There are a whole series of coordinate changes in the 3' half of the env gene of all SFFVp, which sets SFFVp apart from the R-SFFV and the SFFVa. All of these changes which result in amino acid alterations could be of significance to the specific feature of SFFVp, i.e., its unique property to completely replace the requirement for erythropoietin in infected erythroid precursor cells.

The progressive change from a MCFV-type env region to a SFFVp like env gene would suggest that SFFVa is a precursor of SFFVp and thus the ancestral FV-A. This has been repeatedly pointed out by the late C. Friend (Brown et al., 1985). Further alterations of SFFVa (smaller genome size, as well as other alterations), must have followed in the divergent evolution of SFFV. It appears that secondary recombinations were important for the evolution of SFFV as indicated by different points of crossover of SFFVp and SFFVa. The different crossover points in R-SFFV could either indicate its independent evolution or secondary recombination as has been shown for SFFVp with related viral sequences (Mol et al., 1982).

Materials and Methods

Source of Chemicals

Amersham Buchler, Braunschweig, F.R.G.,

Beckmann, Munich, F.R.G.,

Belco, Tecnomara, Ruhberg, F.R.G.,

Boehringer Mannheim, Mannheim, F.R.G.,

BRL/Gibco, Eggerstein, F.R.G.,

DuPont, De Nemours, New England Nuclear Research Products,
Dreieich, F.R.G.,

Flow, Irvine, Scotland,

Greiner, Nurtingen, F.R.G.,

Kulzer, Wehrheim, F.R.G.,

Merck, Darmstadt, F.R.G.,

New England Biolabs, Schwalbach/Taunus, F.R.G.,

Nunc, Wiesbaden/Biebrich, F.R.G.

Pharmacia, Uppsala, Sweden,

Serva, Heidelberg, F.R.G.,

Sigma Chemie GmbH, Diesenhofen, F.R.G.,

Schleicher and Schüll, Dassel, F.R.G..

Cells

F4-6	Krieg et al., 1978
F4+	Krieg et al., 1978
F4N+2	Eisen et al., 1978
B8/3	Krieg et al., 1978
PA317	Miller and Buttimore, 1986
U2 (psi-2)	Mann et al., 1983
RAT1 643/22N	Pragnell et al., 1978
RAT1	Topp, 1981
NIH 3T3	Botchan et al., 1976
A31 (BALBc/3T3)	Aaronson and Todaro, 1968.

Bacterial strains used.

1) CMK603: thr, leu, thi, SupE, recBc, $T_1^r T_6^r$, $r^- m^+$, LacZ m15, △Lac Y, F', pro$^+$.

2) DH1: F^-, endA1, hsd R17, (rk^-, mk^+), supE44, thi-1, λ $^-$, resA1, gyrA96, relA1.

3) HB101: Hsd S 20 (r_B^-, m_B^-), F^-, proAZ, galK2, rpSL20, (Sm^r), rec A13.

4) WM522: Hsd $^-$ rec^-, △(Lac-proAB), thi, SupE, F', proAB lac^q Z △M15.

5) DH5α: F^-, endA1, hsd R17 (rK^- mK^+) SupE44, thi, recA1, gyrA96, relA1, φ80d, LacZ M15.

6) LE392: F^-, hsd R514 (r_K^-, m_K^+) supE44, supF58, LacY1 or △(Lac IZY)6, galK2, galT22, metB1, trpR55, λ $^-$.

7) JM101: SupE, thi, A (Lac proA,B) /F', tra D36, proA,B (r_K^+, m_K^+) $lacI^qZ$ M15.

Plasmids

pUC9 (8)	Vieira and Messing, 1982
pBR322	Bolivar et al., 1977
pSFFVp	Linemeyer et al., 1980
pF-MuLV 57	Oliff et al., 1980
pUC19 (18)	Yanisch-Perron et al., 1985
M13mp19 (18)	Vieira and Messing, 1982
pAG60	Colbère-Garapin et al., 1981
pCDMneo (pmos^{-2})	Stocking et al., 1986
pMPSV3'LTR	Stocking et al., 1985.

Eukaryotic cell culture.

Mammalian cells were generally grown at 37°C in an incubator gassed with 5% (v/v) CO_2 in air of relative humidity greater than 95%. All cells were cultured in GMEM (Glasgows Modified Eagles Medium, Flow Laboratories) supplemented with 10% (v/v) foetal calf serum (FCS, Flow Laboratories), 500 units/ml penicillin and 100 µg/ml streptomycin sulphate.

Cells were grown in either plastic tissue culture flasks (25 cm^2 or 75 cm^2; Greiner) or tissue culture petri dishes (Greiner).

For large scale production of extrachromosomal DNA cells were grown in glass roller bottles (Belco) with a sealed cap at 37°C. Roller bottles were rotated at 0.25 rpm on a Belco roller bottle machine.

Confluent monolayers were prepared for subculturing by removing the culture medium and washing the cells with warm PBS (without Mg^{2+} and Ca^{2+}). Adherent cells were then detached from the culture surface by incubation in PBS (without Mg^{2+} and Ca^{2+}) containing 0.5% trypsin (Seromed) at 37°C. Cells were gently dispersed by continual pipetting before being seeded at the required concentration into new culture flasks containing prewarmed growth medium.

Storage of frozen cells.

Cells were frozen in GMEM containing 20% (v/v) FCS and 10% (v/v) DMSO. Glass ampoules were placed in polystyrene containers to ensure that the temperature decreased slowly and placed at -70°C overnight and then stored in liquid nitrogen.

Transfection of mammalian cells. The calcium phosphate method.

(Graham and van der Eb, 1973).

This method can be used very efficiently for either transient expression of introduced genes or construction of stable transformants. The DNA for transfection was prepared as follows:

5-10 µg of the test plasmid (eventually for 2 transfections, T75 flasks) together with 20-25 µg (and 0.5-1 µg of selection plasmid in co-transfection experiments) of carrier DNA (usually sonicated salmon sperm DNA) was ethanol precipitated overnight at -20°C. The washed, dried DNA pellet was then dissolved in 875 µl sterile deionized H_2O and 125 µl 2 M $CaCl_2$ (sterile filtered and stored at 4°C). To a second tube was added 1 ml of 2x HBS (made from a 10x stock solution: 8.18% (w/v) NaCl, 5.94% (w/v) HEPES, 0.2% (w/v) Na_2HPO_4 sterile filtered and stored at 4°C) which had been adjusted to pH 7.12 (very important) just before use. The DNA solution was added dropwise to the 2x HBS with constant agitation upon which a translucent precipitate immediately formed. This precipitate was used for the transfection.

On the day before transfection (designated day 1) the cells to be transfected were plated at a density of $10^4/cm^2$ in GMEM containing 10% FCS. Twelve hours later the cells were fed and after a further 4 h the DNA precipitate was added to the medium and the cells were returned immediately to the incubator to ensure that the pH did not change. After 4-6 h the cells were washed in serum free medium and glycerol shocked as follows: 1-2 ml per flask of 15% glycerol in HBS was added and incubated for 0.5-2 min at 37°C (depending on the cell type). Cells were then washed once with GMEM containing 10% FCS, before being further incubated in the same medium at 37°C and 5% CO_2 in air.

When it was necessary to select transfected cells for resistance to

Geneticin (G418, Gibco/BRL), cells were cultivated in GMEM, 10% FCS containing 400-2000 µg/ml Geneticin 48 h after transfection. For fast growing cells, flasks were split 1:4 before Geneticin selection. G418 resistant clones usually appeared 10-20 days after beginning selection. Individual clones were picked either with ground glass cloning cylinders or with Q-Tips, depending on the cells being used. Individual clones were grown up in 24 well plates (Greiner) before being transferred to T25 flasks for further culture.

Geneticin transfer assay (GTU-assay).

Forty eight hours prior to the assay RAT1 fibroblasts were seeded at a density of 10^3 cells/well into 24 well tissue culture plates in 1 ml GMEM containing 10% FCS. After 24 h the medium was replaced with GMEM containing 5% FCS, 1% DMSO and 6 µg/ml polybrene. The next day medium was removed and replaced with 1 ml diluent, GMEM containing 2% FCS (which had been filter sterilised (0.45 µm-filter), Flow). After 24 h the virus diluent was removed and the cells were fed with 1 ml GMEM containing 10% FCS and incubated at 37°C, 5% CO_2 in air, for a further 24 h, after which time the medium was replaced with selection medium containing 400 µg/ml G418. Cells were fed every two days until distinct resistant colonies could be recognized 14-21 days following infection. Titres were expressed as Geneticin transfer units/ml (GTU/ml).

Cloning of cells in semi-solid medium.

GMEM containing 30% FCS (37°C) was mixed with melted (50°C) 20% agar (w/v, Gibco/BRL) to give an end concentration of 0.4% agar and incubated at 45°C. Cells to be cloned were resuspended at various densities in the agar cloning medium (45°C) before being plated out (3

ml) into 6 well plates (Greiner). The plates were incubated at 4°C for 5-10 min to allow the agar to set before being incubated at 37°C, 5% CO_2 in air in a humid incubator. After 10-20 days the clones were counted and picked with sterile 100 µl capillary tubes. Cloning efficiency or efficiency of infection of resulting G418 resistant cells was determined in G418 concentrations ranging from 1-2 mg/ml in the above medium.

DMSO induction of Friend cells.

Log phase Friend cells were seeded at 2 x 10^5 cells/ml in GMEM containing 10% FCS at day 0. Twenty four hours later (day 1) 50% of the culture (cells and medium) was replaced with GMEM containing 10% FCS and DMSO (1-1.5% end concentration). On day 3, 50% of the culture was removed and replaced with the above DMSO medium. At this point the cell density was approximately 10^6/ml. On day 5 the cells were stained with benzidine (0.5% benzidine, 15% acetic acid, 8% H_2O_2) to determine the presence of haemoglobin.

Isolation of nuclei from Friend cells.

Log phase cells (1 x 10^8) were washed twice in ice cold PBS (without Ca^{2+} and Mg^{2+}), before being resuspended in 1 ml ice-cold 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 100 mM NaCl, and gently pipetted to give a single cell suspension. NP40 (Nonidet P-40, Sigma) was added (from a 10% stock solution) to give a final concentration of 0.5% and the cells were incubated for 5 mins on ice. The lysis procedure was monitored by phase contrast microscopy to determine the percentage of non-lysed cells. When whole cells could still be seen then the incubation was repeated until >90% of the cells were lysed. The lysed cells were

centrifuged for 5 min at 500g in a Sorvall HB4 rotor at 4°C to pellet the nuclei. To the supernatant (the crude cytoplasmic fraction) was added SDS to a final concentration of 1% and NaCl to 1 M. The nuclei were gently resuspended in 1 ml TE solution (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) before adding SDS and NaCl as above.

These fractions were further processed as described for Hirt supernatants (small scale).

Haemopoietic colony assays. (Fagg and Ostertag, 1982).

Virus-infected mice were used for experiments at 4-16 days post infection when the enlarged spleen was easily detected by palpation. Uninfected animals of a similar age were used as controls. Mice were killed by cervical dislocation, the spleen and both femurs were removed under sterile conditions and the wet weight of the spleen was recorded. The marrow was flushed from both femurs into Iscove's Modified Dulbecco's Medium (IMDM, Boehringer Mannheim) containing 2% FCS and dispersed by repeated passage through a syringe with a 22 gauge needle. The resultant single cell suspension was plated in IMDM containing 10% FCS.

Similarly single cell suspensions from spleen were prepared by gently teasing the spleen apart in IMDM containing 2% FCS. Bone marrow cells were plated out at 1-2 x 10^5 cells/ml and spleen cells at 1-8 x 10^5 cells/ml in the absence or presence of erythropoietin (0.2-0.5 U/ml). The erythropoietin source was a 10x concentrated cell culture supernatant from the cell line IW32, which overproduces this hormone (Tambourine et al., 1983), multi-CSF (5-10% supernatant 10x concentrated) from the cell line WEHI3B (Ymer et al., 1985) and G418 (1.5-2 mg/ml). Cells were plated in a mixture of 1% methylcellulose containing

human transferrin (300 µg/ml, Boehringer Mannheim) and 1% FCS (specially selected as CSF free) together with the above factors.

After 2 days incubation at 37°C in 5% CO_2 the number of colonies arising from CFU-E cells (CFU-E colonies) were counted microscopically. The colonies were confirmed as erythroid by staining with benzidine. Nonstained cultures were further incubated until day 9-14 when CFU-M, G, GM, mixed erythroid and BFU-E colonies were scored.

XC assay.

(Heidi Stuhlmann, pers. comm., Whitehead Institute, Boston, USA, after Rowe et al., 1970).

Virus titration on NIH3T3 or BALB/3T3 cells. On day 1 cells (either NIH3T3 or BALB/3T3) were seeded out in 24 well plates at 0.3 to 1 x 10^4 cells in 1 ml of GMEM containing 10% FCS and 2 µg/ml polybrene. Twenty four hours later the medium was removed and replaced with 0.1 ml of 1:5 dilutions of the viral supernatant to be titrated in PBS containing 2% FCS. The plates were incubated at 37°C, 5% CO_2 in air for 30 min, before 1 ml GMEM containing 10% FCS was added and further incubated for 24 h at 37°C, 5% CO_2 in air. After this period cells were fed by aspirating medium and replacing with an equal volume of the same medium. After a further 48 h the medium was fully aspirated and the cells were irradiated for 20-30 seconds with a UV source placed 60 cm above the cells. To the irradiated cells were added 2 x 10^5 XC cells in 1 ml GMEM containing 10% FCS and incubated for 24 h at 37°C and 5% CO_2 in air after which time the cells were fed as above. After a further 24 h to 48 h the XC-cells were stained. The medium was removed and the cells were washed once with methanol before being fixed for 10 min with methanol. The cells were then washed once with water before being

stained for 10 min in a 1x Giemsa solution (10x solution: 1 g Giemsa stain, Merck, in 54 ml glycerin and 84 ml methanol and filtered through fine filter paper, diluted with water).

Cells were then washed once with water and allowed to dry. Syncytia were monitored either macro- or microscopically according to size. Virus titres were expressed as XC plaques/ml.

Reverse transcriptase assay. (Pragnell et al., 1977).

1 ml of a log phase cell culture supernatant (which had been fed 24 h previously) was centrifuged in an Eppendorf centrifuge to remove cells and either directly assayed or stored at -20°C, a treatment which does not seem to affect reverse transcriptase activity: 10 µl of sample (in duplicate) was pipetted into a microtitre plate to which 20 µl of "Mix" was added before being incubated at 37°C for 1 hour in a moist atmosphere.

The "Mix" comprised 0.3% (v/v) NP40 (Sigma), 0.25 OD units/ml poly(A) $(dT)_{12}$ (Boehringer Mannheim), 0.0125 mM dTTP, 25 µCi/ml, ^{3}H-dTTP (Amersham, 20 Ci/mM), 12.5 mM Tris-HCl (pH 8.0), 2.5 mM dithiothreitol, 0.0125% Triton X 100 (Serva), 25 mM KCl, 6.25 mM Mg-acetate, 0.625 mM Mn-acetate. After the incubation period 22.5 µl of the mixture was pipetted onto a 2 cm diameter DEAE cellulose (Whatman DE/81) filter, and allowed to dry for 5 min at room temperature. The filters were then washed in 5% w/v Na_2HPO_4 four times for 5 min each (10 ml/filter), once for 5 min in H_2O (10 ml/filter) and finally twice for 2 min each in 96% ethanol (1-2 ml/filter). Filters were again allowed to dry at room temperature on aluminium foil before being counted in 5 ml of toluene base scintillation fluid for 5 min.

Spleen focus formation assay.

Mice were injected i.v. with 0.5 ml of the virus diluent into the lateral tail vein. After 11-20 days, animals were sacrificed and the spleens were removed, weighed and then fixed in 70% ethanol. Macroscopic spleen foci were counted and virus titres were expressed as spleen focus forming units (SFFU)/ml. Foci were only counted at the dilution where approximatly 50-100 individual foci could be easily distinguished. Titres were then calculated by multiplying by the appropriate dilution factors.

Histological and cytological techniques.

Mice were killed by cervical dislocation and spleens were dissected. The tissue blocks were fixed with Bouin's or Carnoy's solution. Semi-thin (1µm) sections were embedded in methacrylate (Technovit, Kulzer, Wehrheim) and were stained with toluidine blue, hematoxylin and eosin or Giemsa.

Preparation of competent bacterial cells for transformation by the calcium chloride procedure. (Mandel and Higa, 1970. Hanahan, 1983).

100 ml of L. Broth (1% (w/v) NaCl, 1% Bactotryptone and 0.5% yeast extract (pH 6.8)) in a 500 ml flask was inoculated with 1 ml of an overnight bacterial culture (HB101 or CMK 603, DH5) and incubated at 37°C with vigorous shaking until the cell density had reached approximately 5 x 10^7 cells/ml. The bacteria were then chilled on ice for 10 min and centrifuged at 1000 rpm in a GSA Sorvall rotor for 15 min at 4°C before being resuspended in half of the original culture volume of ice-cold sterile 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0). The gently resuspended cells were then incubated for 15 min in an ice bath

and pelleted by centrifugation as above. The pellet was gently resuspended in 1/15 of the original volume of ice-cold 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0) and dispersed in 0.2 ml aliquots into prechilled Eppendorf tubes and stored at 4°C for 12 h. Cells were either used for transformation or snap frozen in liquid nitrogen after adding glycerol to form a 20% solution before being stored at -70°C.

Transformation of competent cells.

Ligation reactions containing generally 100 ng of vector DNA and equivalent molar ratios of insert were diluted 1:4 before being used for transformation. In general 1-10 ng of DNA were added to 10 µl of TE and pipetted gently into either fresh (or ice thawed-frozen) competent cells, gently mixed and then incubated on ice for 30 min. The cells were then heat shocked at 42°C for 2 min before being cooled on ice for 2 min. At this point the bacteria could either be plated onto antibiotic agar plates (i.e. when subcloning) or incubated at 37°C for 1 hour in 1 ml of SOC medium (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) before plating (for preparing libraries).

Hanahan procedure for the preparation of competent cells.

(Hanahan, 1983, 1985)

This method is very convenient and efficient, giving an average transformation frequency of 10^7-10^8 colonies per microgram of pBR322 DNA. This method is an adaptation of the standard transformation protocol (Hanahan, 1983) to prepare frozen competent cells for storage, except glycerol has been added and potassium acetate substituted for 2(N-morphino)ethone sulphonic acid (MES), along with DTT, which are

inhibitory in the preparation of frozen competent cells.

This method, for the construction of ~~DNA~~ plasmid libraries, was used in conjunction with the bacteria, DH1 and DH5α.

Bacteria were plated out on SOB agar plates (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) and incubated overnight at 37°C. Single colonies were picked and grown up overnight in 10 ml of SOB medium. This overnight culture was then used to inoculate 100 ml of SOB medium in a 1 l Erlenmeyer flask (1:100). The bacteria were incubated at 37°C with moderate agitation until the cell density was approximately 6-9 x 10^7 cells/ml (OD_{550} of 0.4-0.5). The culture was then transferred to 50 ml polypropylene centrifuge tubes and cooled on ice for 15 min. Cells were pelleted at 750g for 15 min at 4°C. After removing all traces of media, the cells were resuspended in 33 ml of 100 mM KCl, 45 mM $MnCl_2 \cdot 4H_2O$, 10 mM $CaCl_2 \cdot 2H_2O$, 3 mM $HACoCl_3$, 10 mM K-Acetate, 10% (w/v) glycerol (pH 6.2), by gently vortexing on a vortex mixer. The resuspended cells were incubated on ice for 15 min, before being pelleted as before, and then resuspended in 8 ml of the above buffer. DMSO was added to a final concentration of 3-5%, mixed gently, and then incubated on ice for 5 min. A second aliquot of DMSO giving an end concentration of 7% was added as before and the cells were incubated for 15 min on ice. 200 µl aliquots were pipetted into chilled 1.5 ml Eppendorf tubes, before flash freezing in liquid nitrogen. The cells were stored at -70°C until use.

Use of competent cells.

Tubes were removed from -70°C and allowed to thaw on ice, until the cell suspension was just liquid. DNA was added (in a volume of <10 µl),

and the cells were gently mixed, before incubating on ice for 45 min. Cells were heat shocked in a 42°C water bath for 90 sec, and then chilled on ice. 800 µl of SOC medium was added and incubated at 37°C with moderate agitation (150 rpm) for 60 min before plating.

Preparation of bacteriophage lambda stocks.

Bacteriophage from a single plaque were transferred with a sterile Pasteur pipette into 1 ml of phage buffer SM (50 mM Tris-HCl (pH 7.5), 0.01% gelatin, 0.58% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$), 1 drop of chloroform was added, and the solution was mixed on a vortex mixer. After removal of the chloroform by low speed centrifugation, 0.1 to 0.2 ml of phage suspension was mixed with 0.1 ml of fresh cells (prepared by growing a 1:20 dilution of an overnight culture for 2 h and then resuspending the cells in a half volume of 10 mM $MgSO_4$). After allowing the phage to adsorb to the cells for 10 min at room temperature, 2.5 ml of TOP agar (L. broth + 0.8% agar) were added and the mixture was poured onto a fresh agar plate. Confluent lysis was observed after 6-10 h at 37°C. The agar was overlaid with 3 ml of phage buffer for 4-6 h at 2°C. This was then decanted, 2 drops of chloroform were added and the suspension titred and stored at 4°C. Titres varied from 5 x 10^8 to 5 x 10^{11}/ml depending on the phage and the bacterial host used.

Packaging of recombinant DNA into phage heads in vitro.

Packaging extracts were prepared essentially as described by Maniatis et al. (1982). Packaging in vitro was carried out as follows: the frozen packaging extracts (stored at -70°C) were thawed on ice. The freeze/thaw lysate, which thaws first, was transferred to the still frozen sonicated lysate and then the DNA to be packaged (up to 1 µg in

TE and 10 mM $MgCl_2$) was added to the mixture. This was incubated at room temperature for 60-120 mins. After this time 0.5-1 ml of phage storage buffer (SM) and a drop of chloroform was added and gently mixed on a vortex mixer. Debris was removed by brief centrifugation in an eppendorf centrifuge (30 sec). Titres of packaged phage were determined as described previously.

Plasmid DNA preparation.

Plasmid DNA was isolated using an adaptation of the method described by Clewel and Helinski (1969). E.coli strains carrying plasmids were plated onto appropriate antibiotic agar plates so as to obtain single colonies. 10 ml cultures from a single colony were grown to saturation overnight at 37°C and then used to inoculate 500 ml of the appropriate antibiotic containing medium in a 2 liter Erlenmeyer flask. These cultures were then grown at 37°C for 12 h with vigourous shaking. The cells were harvested by centrifugation at 6000 rpm for 5 min at 4°C in a Sorvall GS-3 rotor. The cell pellet was resuspended in 6 ml of ice cold 25% sucrose, 50 mM Tris-HCl (pH 8.0), and transferred to a polypropylene Oak Ridge tube on ice. 0.6 ml of freshly prepared lysozyme (5 mg/ml, Sigma) in 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA were added, mixed gently by inversion and incubated for 5 min on ice. 2.25 ml of 250 mM Tris-HCl (pH 8.0), 250 mM EDTA stop buffer were added, gently mixed and incubated for a further 5 min on ice. After addition of 9 ml 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100, and gentle mixing, lysis of the bacterial spheroplasts was indicated by an increase in viscosity at which point mixing was stopped. The lysate was cleared by centrifugation at 20,000 rpm for 1 hour at 4°C in a Sorvall SS34 rotor. The supernatant was decanted

carefully into a 50 ml polyallomer tube containing 29 g CsCl (Gibco/BRL). The volume was made up to 27 ml with H_2O and 0.5 ml ethidium bromide (5 mg/ml) was added and the solution was transferred to a 39 ml quickseal vti tube (Beckman) through a large bore syringe. The tube was filled up with 0.984 g/ml CsCl in H_2O, sealed and centrifuged for 18 h at 45,000 rpm at 20°C in a Beckman vti 50 rotor. Supercoiled plasmid DNA was collected in a 5 ml syringe by side puncture and transferred to a small (6 ml) Beckman quick seal vti tube and filled up with the CsCl solution described above. Tubes were sealed and centrifuged at 65,000 rpm for 5 h at 20°C in a Beckman vti 65 rotor. Supercoiled plasmid DNA was removed by side puncture and the ethidium bromide was removed by repeated extraction with isobutanol partially saturated with water and the aqueous phase was dialysed against several changes of TE solution. The DNA was precipitated by addition of 0.1 volume 3 M Na-acetate (pH 5.6) and 2.5 volumes of ethanol. After at least 2 h at -20°C the precipitate was collected by centrifugation at 10,000 rpm for 10 min at 4°C in a Sorvall HB-4 rotor. Following brief drying under vacuum, the pellet was resuspended in 0.5-1 ml TE solution.

Small scale preparation of plasmid DNA.

The rapid small scale preparation of plasmid DNA from recombinant clones was that of the alkaline lysis method described by Maniatis et al. (1982), except for minor modifications.

2 ml cultures of E.coli strains containing plasmids were grown for 6 h to overnight at 37°C in L. Broth containing the appropriate antibiotic. The cells were pelleted by centrifugation in an Eppendorf microfuge for 2 min at room temperature and then resuspended in 100 µl

of 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA at room temperature. 200 µl of freshly prepared 0.2 M NaOH, 1% (w/v) SDS were added and the mixture gently mixed. 150 µl of precooled 5 M K^+ 3M acetate$^-$ solution was added immediatly and after brief gentle mixing was incubated for 5 min on ice. The resulting precipitate was removed by centrifugation for 5 min in an Eppendorf microfuge at room temperature and the supernatant was transferred to a fresh tube. This aqueous phase was then extracted with an equal volume of phenol/chloroform (1:1) and after centrifugation for 2 min in an Eppendorf microfuge was removed to a fresh tube. The plasmid DNA was precipitated by addition of 800 µl of absolute ethanol and allowed to stand at room temperature for 5 min. The DNA was collected by centrifugation for 5 min at room temperature in an Eppendorf microfuge. The supernatant was carefully aspirated and the pellet was washed with 70% (v/v) ethanol, dried under vacuum, resuspended in 50 µl TE solution and stored at -20°C.

Large scale isolation of bacteriophage DNA.

A suspension of phage was mixed with 0.5 ml fresh plating cells to give a m.o.i. of 1-10. After allowing the phage to adsorb to the cells for 15 min at room temperature, the suspension was inoculated into 500 ml L. broth containing 10 mM $MgSO_4$, 0.2% maltose and incubated overnight at 37°C with vigorous shaking. To the resultant phage lysate, chloroform (2 ml/l) and NaCl (40 g/l) were added plus RNase and DNase to 1 µg/ml. The lysate was incubated at room temperature for at least 1 hour and then clarified by centrifugation at 8000 rpm in a Sorvall GS-3 rotor for 10 min at 4°C. Solid PEG 6000 was added to the supernatant to give a final concentration of 10% (w/v) and swirled to

dissolve. The opaque solution was then left at 4°C for at least 1 hour to precipitate the phage. The phage was pelleted by centrifugation at 8000 rpm for 20 min in a Sorvall GS-3 rotor at 4°C and resuspended in 9 ml of phage buffer overnight at 4°C. The volume of phage suspension was made up to 10 ml with phage buffer and then 7.1 g CsCl was added. The phage was purified by equilibrium centrifugation in a Beckman Ty65 rotor at 50,000 rpm for 16-18 h. The phage band was collected in a 2 ml syringe by side puncture and the CsCl was removed by dialysis against several changes of TE. The phage suspension was extracted 3 times with neutral phenol (equilibrated with Tris base and hydroxychinoline) and once with chloroform before dialysis against several changes of TE solution. The DNA was stored at 4°C.

Small scale preparation of lambda DNA.

This method was used to analyse clones found to be positive upon plaque hybridisation.

0.1 ml of the phage suspension was incubated for 20 min at 37°C with 0.2 ml of host bacteria and 0.2 ml of a 10 mM $MgCl_2$, 10 mM $MgSO_4$ mixture. 2 ml of prewarmed LB (containing 0.2% maltose) was added and incubated overnight at 37°C with vigorous shaking. After this period lysis was visible and was further aided by the addition of a drop of chloroform and incubation for 5 min at 37°C with vigorous shaking. Debris and non-lysed bacteria were pelleted by centrifugation at 6000 rpm for 15 min in a Sorvall HB4 rotor at 4°C. The supernatant was mixed with 0.4 ml 0.25 M EDTA (pH 8.0), 0.4 ml 0.5 M Tris-HCl (pH 9.5) and 0.4 ml 3% (w/v) SDS and incubated at 65°C for 15 min. 0.4 ml 5 M K-acetate was added and incubated on ice for 10 min before being

centrifuged for 15 min at 6000 rpm in a Sorvall HB4 rotor at 4°C. The clear supernatant was then extracted twice with equilibrated phenol and twice with a phenol/chloroform mixture before precipitating the DNA by addition of two volumes of absolute ethanol and 30 min at -70°C. The DNA was pelleted by centrifugation at 8000 rpm for 30 min in a Sorvall HB4 rotor at 4°C. The washed, dried DNA was then resuspended in 200 µl TE and stored at 4°C.

Preparation of extrachromosomal DNA (Hirt extraction). (Hirt, 1967).

Large scale preparation.

Cells (ca. 10^7-10^8) were pelleted by centrifugation at 1000 rpm for 10 min at 4°C in a Sorvall GS-3 rotor. The cells were washed once in cold PBS and pelleted as above before being resuspended at 3 x 10^8 cells/ml in 10 mM Tris-HCl (pH 8.0), 10 mM EDTA. SDS (10% w/v) was added to a final concentration of 0.6% and proteinase K (20 mg/ml in H_2O) to 200 µg/ml. This mixture was then incubated at 60°C for 30 min. 5 M NaCl was added to give an end concentration of 1 M and incubated at 4°C overnight. High molecular weight DNA was pelleted by centrifuging the lysate at 17,000 rpm for 60 min in a Sorvall SS34 rotor at 4°C. The resulting cleared lysate was further digested with 50 µg/ml proteinase K for 1 hour at 60°C before being extracted twice with equilibrated phenol, twice with a phenol/chloroform mixture and once with chloroform. DNA was precipitated by addition of two volumes of ethanol and overnight incubation at -20°C. The washed DNA pellet (70% ethanol) was dried under vacuum and dissolved in TE.

Before electrophoresis DNA was treated with RNase A to remove RNA contamination.

Small scale preparation .

(H. Weiher, pers. commun., Whitehead Institute, Boston).

1 x 10^6 cells were washed in PBS and then resuspended in 900 µl 10 mM Tris-HCl (pH 8.0), 10 mM EDTA in an Eppendorf microfuge tube. 100 µl of 10% SDS (w/v) was added together with 200 µg/ml proteinase K (20 mg/ml stock solution in H_2O) and incubated at 60°C for 20 min. To this was added 200 µl of 5 M NaCl and incubated at -20°C for 20 min. The precipitated high molecular weight DNA was pelleted by centrifugation in an Eppendorf microfuge in the cold room for 10 min. The supernatant was then extracted twice with a phenol/chloroform mixture and once with chloroform. DNA was precipitated by addition of two volumes of ethanol and incubation at -70°C for 20 min. Pelleted, washed, dried DNA was resuspended in TE solution.

Preparation of mammalian genomic DNA.

Either homogenised tissue or tissue culture cells were washed once in PBS and then resuspended in 10 mM Tris (pH 8.0), 10 mM EDTA. To this suspension was added SDS (10% w/v stock solution) to give a final concentration of 1% and proteinase K at 100 µg/ml. Lysed cells were then incubated at 37°C overnight with constant gentle agitation, before being gently extracted twice with equilibrated phenol, twice with a phenol/chloroform mixture and once with chloroform. The genomic DNA was precipitated by addition of two volumes of isopropanol and 0.1 volumes of 5 M NaCl. The precipitate was collected by spooling the DNA onto a closed-end Pasteur pipette. The DNA was briefly washed in 70% ethanol before being allowed to dry briefly at room temperature. The dried DNA was dissolved in TE solution and stored at 4°C.

RNA preparation:

In all procedures involved in preparing RNA and any experiment using RNA, precautions against ubiquitous ribonuclease were taken. All glassware and DTFE Oak Ridge centrifuge tubes were heated in a dry oven at 160°C for at least 2 h. Polypropylene microfuge tubes and disposable pipette tips were used directly from the manufacturer's sealed packets. When possible, new containers of chemicals were used. All buffer solutions were autoclaved before use.

Total RNA.

The method used was that of Auffray and Rougeon (1981). Fresh tissue, usually cultured cells, or finely ground frozen tissue were placed in 10 volumes or more of 6 M urea, 3 M LiCl and homogenised in a polytron for 1 min. The RNA was precipitated out by overnight incubation at 4°C and then pelleted by centrifugation at 12,000 rpm for 30 min at 0°C in a Sorvall SS34 rotor. The RNA pellet was then resuspended in 8 ml 6 M urea, 3 M LiCl and again centrifuged for 30 min at 12,000 rpm, 0°C in a Sorvall SS34 rotor. The resulting pellet was then dissolved in 10 mM Tris-HCl (pH 7.5), 0.5% SDS, 50 µg/ml proteinase K (Merck) and incubated at 37°C for 15 min. This aqueous solution was then extracted 3 times with equilibrated phenol, twice with a phenol/chloroform mixture and once with chloroform. The RNA was then ethanol precipitated and dissolved in H_2O and stored at -70°C.

PolyA⁺ RNA.

This was prepared according to the method of Aviv and Leder (1972). The total RNA pellet was dissolved in a minimal volume of 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5) and 0.5% SDS (binding buffer). This solution

was passed three times over a small (less than 1 ml) oligo (dT) cellulose column (Pharmacia Type 7) preequilibrated with binding buffer. Unbound material, including residual DNA and proteins, was removed by a 30 ml wash with binding buffer. Bound material was eluted with 10 ml 10 mM Tris-HCl (pH 7.5) and 0.05% SDS (eluting buffer). The eluate was adjusted to 0.5 M NaCl and twice reapplied to the column. The column was washed with 20 ml binding buffer, and bound material was again eluted with 5 ml eluting buffer and ethanol precipitated before being dissolved in H_2O after drying. RNA to be used for any purpose was thawed, used immediately and refrozen as infrequently as possible.

Enzymatic reactions:

Restriction enzyme analysis.

DNA samples were digested with restriction enzymes in volumes ranging from 10-500 µl essentially according to the manufacturers instructions using the reaction buffers depicted in Table 1.

Dephosphorylation.

The terminal 5' phosphates were removed from linearized vector DNAs by treatment with calf intestinal phosphatase (CIP). The DNA was digested to completion with a chosen restriction endonuclease, extracted once with a phenol/chloroform mixture prior to ethanol precipitation. The DNA was dissolved in a minimum volume of TE, 5 µl of 10x CIP buffer (Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine) were added and the volume was made up to 50 µl with deionised water. 0.01 units of CIP per pmole of DNA 5' ends were added. DNA with protruding 5' termini were incubated at 37°C for 30 min, a

Table 1. Buffers used for digestion of DNA with various restriction enzymes

The conditions varied only in NaCl concentration. All buffers contained 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 100 µg/ml bovine serum albumin.

+++ represents 30-100% digestion activity.

++ represents 10-30% digestion activity.

+ represents < 10% digestion activity.

* not recommended because of star activity.

ENZYME	0mM NaCl	50mM NaCl	100mM NaCl	150mM NaCl
Ase II	+	+ +	+ +	+
Acc I	+ + +	+ + +	+	+
Aha II	+	+ +	+ + +	+ + +
Aha III	+	+ +	+ + +	+ + +
Alu I	+	+ + +	+ + +	+ +
Apa I	+ + +	+ + +	+ + +	+ +
Ava I	+ + +	+ + +	+ +	+
Ava II	+ + +	+ + +	+ +	+
Bal I	+ + +	+ +	+ +	+
BamHI			+	+ + +
Ban I	+ + +	+ + +	+ +	+ +
Ban II	+ + +	+ + +	+ +	+ +
Bbv I	+ + +	+ + +	+ +	+ +
Bcl I	+	+ + +	+ + +	+
Bgl I	+	+ + +	+ + +	+
Bgl II	+ +	+ + +	+ + +	+ + +
Bsm I	+ + +	+ + +	+ + +	+ + +
Bsp 1286	+ + +	+ + +	+ +	+
BssHII	+ + +	+ + +	+ + +	+ + +
BstE II	+	+ +	+ + +	+ + +
BstN I	+ +	+ + +	+ + +	+ + +
BstX I	+ +	+ + +	+ + +	+ + +
Cla I	+ + +	+ + +	+ + +	+ + +
DdeI	+ +	+ + +	+ + +	+ + +
Dpn I	+	+ + +	+ + +	+ + +
Dra I	+ +	+ + +	+	+
EcoR I	+	+ + +	+ + +	+ + +
EcoR V	+	+	+ +	+ + +
FnuDII	+ + +	+ + +	+ +	+
FnuDII	+ + +	+ +	+ +	+

ENZYME	0mM NaCl	50mM NaCl	100mM NaCl	150mM NaCl
Fok I	+ + +	+ + +	+ + +	+ + +
Fsp I	+	+ + +	+ +	+ +
Hae II	+ + +	+ + +	+ + +	+ +
Hae III	+ + +	+ + +	+ + +	+ +
Hga I	+ + +	+ + +	+	+
HgiA I	+	+	+ +	+ + +
Hha I	+	+ +	+ + +	+ + +
Hinc II	+ +	+ + +	+ + +	+ + +
Hind III	+	+ +	+ + +	+ +
Hinf I	+	+ + +	+ + +	+ + +
HinP I	+ +	+ + +	+ + +	+ + +
Hpa I	+	+ + +	+ + +	+
Hpa II	+ +	+ + +	+ +	+
Hph I	+ +	+ + +	+ + +	+
Kpn I	+	+ +	+	+
Mbo I	+	+ + +	+ + +	+ + +
Mbo II	+ +	+ + +	+ + +	+ + +
Mlu I	+	+ +	+	+ +
Mnl I	+ +	+ + +	+ +	+
Msp I	+ +	+ + +	+ + +	+ + +
Mst I	+	+ + +	+ + +	+ + +
Mst II	+	+ + +	+ + +	+ + +
Nae I	+ +	+ + +	+ + +	+
Nar I	+ +	+ + +	+ +	+
Nci I	+ +	+ + +	+ +	+
Nco I	+	+	+ +	+ + +
Nde I	+	+	+ +	+ + +
Nhe I	+ +	+ + +	+ + +	+ +
Nla III	+	+	+	+
Nla IV	+	+	+	+

ENZYME	0mM NaCl	50mM NaCl	100mM NaCl	150mM NaCl
Not I		+ + +	+ + +	+ + +
Nru I	+	+ + +	+	+ + +
Nsi I	+ +	+ + +	+ +	+ +
Pst I	+ +	+ + +	+ +	+
Pvu I	+	+ +	+ +	+ +
Pvu II	+ +	+ + +	+ +	+ +
Rsa I	+ +	+ + +	+ +	+ +
Sac I	+ + +	+ + +	+ +	+
Sal I			+ +	+
Sau3A I	+ +	+ + +	+ +	+ +
Sau96 I	+ +	+ + +	+ +	+ +
Sca I	+	+ + +	+ +	+ +
ScrF I	+ +	+ + +	+ +	+ +
SfaN I	+	+	+ +	+ + +
Sma I	+	+	+	+
SnaB I	+ +	+ + +	+ +	+
Spe I	+ +	+ + +	+ +	+ +
Sph I	+	+	+	+ +
Sst I	+ +	+ + +	+ +	+ +
Stu I	+ +	+ + +	+ +	+ +
Taq I	+ +	+ + +	+ +	+ +
Tth111 I	+ +	+ + +	+ +	+ +
Xba I	+	+ + +	+ +	+ +
Xho I	+	+ + +	+ +	+ +
Xho II	+ +	+ + +	+ +	+
Xma I	+ +	+ + +	+ +	+
Xma III	+ +	+ + +	+ +	+ +
Xmn I	+ + +	+ + +	+	+

+Not recommended because of star activity

second aliquot of CIP was added and the reaction incubated for a further 30 min. Recessed 5' or blunt ends were incubated for 15 min at 37°C and then 15 min at 56°C, a second aliquot of CIP was added and the incubations repeated. Reactions were terminated by addition of 40 µl H_2O, 10 µl of STE and 5 µl of 10% (w/v) SDS and heated to 68°C for 15 min. The reactions were extracted twice with phenol/chloroform and once with chloroform before the DNA was ethanol precipitated together with 1 µg of glycogen carrier. CIP was obtained from Boehringer Mannheim.

Filling in.

Recessed 3' ends of double stranded DNA were filled in using the Klenow fragment of E.coli DNA polymerase I. Final reaction conditions were 0.6 mM $MgCl_2$, 6.6 mM Tris-HCl (pH 8.5), 2 mM dNTPs, 50 µg/ml DNA and 1 unit Klenow fragment of E. coli DNA polymerase I (Boehringer Mannheim). The reaction mixture was incubated at room temperature for 30 min, extracted once with phenol/chloroform, and the DNA ethanol precipitated prior to ligation. This method was also used to label ends by addition of ^{32}P dNTPs to the reaction.

Labelling of 5' ends with T4 polynucleotide kinase

This method is used to label DNA molecules with 5' protruding termini by catalysing the transfer of the γ-phosphate of ATP to a 5' OH terminus in DNA (or RNA).

Digested, dephosporylated and ethanol precipitated DNA (1-50 pmoles of 5' ends) was resuspended in 10 µl of 10x kinase buffer (0.5 M Tris-HCl (pH 7.6), 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA) and 24 µl H_2O. To this mixture 150 µCi (3000 Ci/mmol, 15 µl) ($\gamma^{32}P$)ATP (Amersham) was added followed by 10-20 units (1-2 µl) T4 polynucleotide

kinase (New England Nuclear). The mixture was incubated at 37°C for 30 min, before adding 2 µl of 0.5 M EDTA and extracting once with phenol/chloroform and subsequently ethanol precipitated. The free nucleotides were separated (when needed) from the labelled DNA by spin column chromatography with Sephadex G-50.

Digestion of linearised DNA with Bal 31 nuclease.

DNA to be digested with Bal 31 was ethanol precipitated and then dissolved in 500 µg/ml (in water) of BSA. To this DNA solution was added an equal volume of 2x Bal 31 buffer (24 mM $CaCl_2$, 24 mM $MgCl_2$, 400 mM NaCl, 40 mM Tris-HCl (pH 8.0), 2 mM EDTA). This mixture was incubated at 30°C for 3 minutes. An appropriate amount of Bal 31 was added and incubated at 30°C. At time intervals aliquots were removed from the reaction and made 20 mM EGTA on ice. The conditions used were that appr. 10 bp/min/end of double stranded DNA were digested.

Ligation.

For plasmid construction experiments, the DNA concentration in the ligation reaction was 100 µg/ml and the target to vector molar ratio was 3:1. The target and vector DNAs were mixed together with 5 µg glycogen carrier and were ethanol precipitated. The nucleic acids were dissolved in 8 µl TE and 1 µl of 10x ligation buffer (100 mM Tris-HCl (pH 7.5), 0.1 M $MgCl_2$, 0.15 M DTT, 10 mM spermidine). 10 mM ATP was added with 1 unit of T4 DNA ligase (Boehringer Mannheim). The reaction was incubated at 14°C overnight (room temperature for blunt ends).

Labelling of nucleic acids:

Nick-translation. (Rigby et al., 1977).

Final reaction conditions were 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 µM unlabelled dNTPs, 100 mM dithiothreitol, 50 µg/ml BSA pentax fraction V, 1 µg DNA, 50-100 µCi α-^{32}P dNTP. The reaction volume was made up to 44 µl with H_2O. 0.5 µl of a freshly diluted DNAse solution (0.1 µg/ml, stock solution 1 mg/ml) together with 5 units of E.coli DNA polymerase I was added and mixed.

The reaction was incubated at 14°C for 1 hour and then the labelled DNA was separated from the unincorporated nucleotides by gel filtration through a Sephadex G-50 (Pharmacia) spin column (equilibrated with TE) in a 2 ml syringe at 1000 rpm for 5 min in a bench centrifuge. The DNA was denatured by boiling for 5 min, cooled on ice, then diluted as required into hybridisation reactions.

Routinely 50-70% of labelled dNTP was incorporated into the G-50 excluded fraction, giving a final specific activity of greater than 10^8 cpm/µg.

Oligonucleotide labelling of DNA restriction endonuclease fragments. (Feinberg and Vogelstein, 1984).

This method was used to label DNA fragments excised from gels to high specific activity with α-^{32}P. DNA fragments from restriction endonuclease digests were electrophoretically separated and isolated either by electroelution or NA45 paper. The labelling reaction was carried out at room temperature in the following way. 10-50 ng of fragment DNA were denatured by boiling for 10 min in 30 µl of H_2O. This was then incubated at 37°C for 20-30 min, before adding 10 µl of OLB

buffer. OLB buffer is made from the following components: Solution O: 1.25 M Tris-HCl (pH 8.0), 0.125 M $MgCl_2$, stored at 4°C. Solution A: 1ml solution O and 18 µl 2-mercaptoethanol, 5 µl dATP, 5 µl dTTP, 5 µl dGTP (0.1 M in 3 mM Tris-HCl (pH 7.0), 0.2 mM EDTA), stored at -20°C. Solution B: 2 M HEPES, titrated to pH 6.0 with 4 M NaOH, stored at 4°C. Solution C: Hexadeoxyribonucleotides (Pharmacia) evenly suspended in TE at 90 OD_{260} units/ml, stored at -20°C. A:B:C was mixed at a ratio of 10:25:15 to make OLB and was stored as 10 µl aliquots at -20°C. To this was then added 1 µl BSA (20 mg/ml, Sigma, pentax fraction V), 50-100 µCi α-^{32}P-labelled dCTP (Amersham Buchler), 2 units of Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim). Incubation was continued at room temperature for 2-16 h, then 200 µl of the stop solution (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.25% (w/v) SDS and 1 µM dCTP) were added. The solution was extracted twice with a phenol/chloroform mixture and once with chloroform before being denatured by boiling for 5 min. Specific activities of greater than 10^9 cpm/µg were routinely obtained.

Quantitation of nucleic acids:

Spectrophotometric assays.

Samples were diluted in TE and the absorbance at 260 nm was measured. Concentrations were calculated assuming that an OD_{260} of 1.0 corresponds to 50 µg/ml of double stranded DNA or 40 µg/ml of RNA.

Radiometric assays.

DNA synthesis reactions (nick-translation etc.) were monitored by percentage incorporation of ^{32}P-labelled nucleotide into acid insoluble

material. An aliquot (0.01-0.1 reaction volumes) was diluted in 0.1-1.0 ml TE. An aliquot of this was dried onto a Whatman GF/C filter, washed in acid solution followed by ethanol and dried. All filters were counted in toluene-based scintillation fluid. The yield of DNA synthesized was calculated from the percentage incorporation and from the known concentration of DNA the specific activity was calculated.

Electrophoretic techniques:

Neutral agarose gels.

0.4-2% (w/v) horizontal agarose gels were prepared in electrophoresis buffer, TAE buffer (40 mM Tris-HCl (pH 8.1), 20 mM Na-acetate, 2 mM EDTA) and subjected to electrophoresis in the same buffer in the presence of ethidium bromide (1 µg/ml) at 1.5 V/cm for 6-18 h. 0.2 volumes of gel loading buffer (10x: 0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll (type 400) in water) were added to samples prior to loading. This method was used for fine restriction mapping of plasmid constructs and genomic DNA. For other purposes we used an electrophoresis buffer consisting of 0.5x TBE (1x: 90 mM Tris, 2.5 mM EDTA, 90 mM boric acid (pH 8.3)) as this could be used at higher voltages (5-10 V/cm).

Isolation of DNA fragments after restriction enzyme digestion.

Specific DNA digestion fragments (to be used as ^{32}P probes or for subcloning etc.) were isolated by either one of the following methods.

1) Electroelution.

After digestion the fragments were separated on neutral agarose gels

(0.5-2%) in 0.5x TBE solution containing ethidium bromide, until the required fragment(s) was (were) well separated from other fragments. An agarose block (as small as possible) containing the required fragment was placed into dialysis tubing containing a minimal volume of 0.25x TBE solution (200-500 µl) and sealed without trapping any air bubbles. The dialysis tubing was submerged in 0.25x TBE in a mini-gel electrophoresis chamber and the fragment was eluted from the agarose block into the dialysis bag by electrophoresis at 200 V for 1 hour. After this period the poles were reversed and a voltage was applied (100-200 V) for 30 sec to remove DNA from the dialysis tubing wall. The 0.25x TBE and DNA solution in the dialysis tubing was removed and extracted twice with a phenol/chloroform mixture before being ethanol precipitated.

2) NA45 paper. (Ken Burtis, pers. comm., Stanford, USA).

After digestion and separation of DNA fragments as described above, a small strip of NA45 paper (Schleicher and Schüll) corresponding to the length of the required band was placed in a slit directly in front of it. The DNA was then electrophoresed onto the NA45 paper, a process which was easily monitored by UV illumination. The DNA was eluted from the NA45 paper by incubation for 2 h at 68°C in 400 µl of 1 M NaCl, 50 mM arginine (free base). This DNA containing solution was extracted twice with a phenol/chloroform mixture before being ethanol precipitated.

RNA glyoxal gels.

These gels contained 1-1.5% (w/v) agarose and were cast in MOPS (10 mM MOPS, 2.5 mM Na-acetate, 0.25 mM EDTA (pH7.0)) buffer without

ethidium bromide. Gels were subjected to electrophoresis in the same buffer at 1-2 V/cm for 12-18 h with constant recircularisation of the buffer. Samples to be analysed by electrophoresis were prepared as follows: 10-20 µg of total or polyA$^+$ RNA in 15.3 µl H_2O was added to 25 µl DMSO, 2.5 µl 0.2 M Na_2HPO_4/NaH_2PO_4 (pH 7.0) and 7 µl 7 M deionized glyoxal. The reaction was incubated at 50°C for 60 min before adding 0.1 volume of loading buffer.

All glass and plexiglass ware were treated with 0.4 M NaOH for 30 min and then washed thoroughly with distilled water prior to use.

Size markers used for electrophoresis.

Restriction enzyme digests of plasmid or bacteriophage lambda DNA were used.

SDS polyacrylamide gel electrophoresis.

Polypeptides were resolved on a 10-30% gradient of polyacrylamide gel using the buffer system of Laemmli (1970) essentially according to Cook et al., (1974). Electrophoresis was at 80 V for 12-16 h until the bromophenol blue or the visible monomer cytochrome C band had reached the bottom of the gel.

Preparation of filters for DNA gel transfer hybridisation (Southern blotting).

DNA was transferred from neutral agarose gels to filters as described by Southern (1975) but incorporating some of the modifications of Wahl et al., (1979). Gels were treated for 15 min with five gel volumes of 0.25 M HCl. They were then rinsed briefly with water, treated for 30 min with five gel volumes of 0.4 M NaOH, 0.6 M NaCl and then

neutralised with five volumes of 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4).

Gene Screen (DuPont) sheets were cut to the exact size of the gel and then wetted in water and allowed to soak in 10x SSC (1.5 M NaCl, 150 mM Na-citrate) for 15 min. DNA was transferred to these overnight using 10x SSC. After transfer the membrane was immersed in an excess of 0.4 M NaOH for 30-60 seconds to ensure complete denaturation of immobilised DNA. The membrane was then immersed in an excess of 0.2 M Tris-HCl (pH 7.5), 2x SSC, before being allowed to dry at room temperature.

An alternative transfer method for nylon nitrocellulose membranes (Reed and Mann, 1986) was also used throughout the course of this work.

Alkaline blotting with depurination.

The gel was soaked in 2 volumes of 0.25 M HCl with gentle agitation until the bromophenol blue turned yellow. The gel was rinsed briefly with distilled water. The normal transfer apparatus was set up during this procedure, except that the transfer solution was 0.4 M NaOH. Transfer was continued for 2-18 h, after which time the filter was washed in 2x SSC before being allowed to dry at room temperature.

Preparation of filters for RNA gel transfer hybridisation (Northern blotting).

The transfer of RNA from MOPS glyoxal gels was essentially the same as for DNA except that the gel was not treated after electrophoresis. Transfer onto Gene Screen filters was conducted with 10x SSC overnight. After transfer the membrane was immersed in 50 mM NaOH for approximately 10-20 seconds to reverse the glyoxal reaction. The membrane was then incubated for 30 seconds in 1x SSC and 0.2 M Tris-HCl

(pH 7.5), before allowing the membrane to dry at room temperature.

Hybridisation of DNA bound to Gene Screen Plus nylon membranes.

Dry membranes were prehybridised in a sealed plastic bag at 68°C for at least 15 min in 5-20 ml of the following solution: 1% SDS, 1 M NaCl and 10% dextran sulphate (Pharmacia Fine Chemicals).

After the prehybridisation step, 0.5-1 ml of the following solution was added to the bag containing the prehybridisation buffer and the membrane: denatured herring sperm DNA (>100 µg/ml) and denatured radioactive probe at a final concentration of less than 10 ng/ml (or 1 x 10^8 to 10^9 dpm/ml). The bag was resealed and incubated for 12-18 h at 68°C with constant agitation. After this period the membrane was removed and washed twice in 100 ml of 2x SSC at room temperature for 5 min with constant agitation, then twice in 200 ml of a solution containing 0.1x SSC and 1.0% SDS at 65°C for 30-60 min with constant agitation. Finally the membrane was washed twice in 100 ml 0.1x SSC at room temperature with constant agitation, before being blotted between two paper towels. Membranes were wrapped in Frappan and exposed to X-ray (DuPont) film at -70°C with intensifying screens.

Filters were rehybridised as above after the original probe had been removed by the following treatment: the membrane was incubated for 30 min at 42°C with gentle agitation in 100-200 ml 0.4 M NaOH. After this the membrane was incubated in 100-200 ml 0.1x SSC, 0.1% SDS, 0.2 M Tris-HCl (pH 7.5), at 42°C for 30 min with gentle agitation, before being autoradiographed to see if sufficient probe had been removed.

Hybridisation of RNA bound to Gene Screen Plus nylon membranes.

The dry membrane was prehybridised in 10-20 ml of the following

solution: 50% deionised formamide, 1% SDS, 1 M NaCl and 10% dextran sulphate in a sealable plastic bag for at least 15 min at 42°C with constant agitation. After this preincubation denatured herring sperm and radioactive probe was added at the same concentration as for DNA hybridisation. The membrane was then hybridised at 42°C for 18-24 h with constant agitation.

Washing of the membrane was identical to the DNA procedure except that the temperature was decreased to 56°C.

To remove probe for rehybridisation, the membrane was washed twice to four times in 500-600 ml of boiling 0.01% SDS, 0.01x SSC. Removal of the probe was monitored by autoradiography.

Screening of bacterial colonies with radioactive probes. (Grunstein and Hogness, 1975).

In this method, 5000-10,000 colonies/plate were screened after transformation and plating out onto 20x20 cm (Nunc) disposable Petri dishes.

After 12-16 h incubation at 37°C, so that the colonies were approximately 0.5-1 mm in diameter, plates were removed and incubated at 4°C for 15 min. Colonies were lifted onto nitrocellulose membranes (Schleicher and Schüll) by placing the filter onto the colony covered dishes and marking orientation positions with India ink. Replicas of these lifts were produced by layering a second nitrocellulose filter onto colonies on the first filter and pressing firmly to equally distribute the bacteria to both filters. The filters were separated and the DNA was liberated from the bacteria by the following method.

The filters were layered colony side up onto Whatman 3 MM paper saturated with 10% SDS and incubated for 3 min at room temperature.

After this the filters were transferred to 3 MM paper saturated with 0.5 M NaOH, 1.5 M NaCl for 5 min and then to 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for a further 5 min. Finally the filters were washed in 2x SSPE (360 mM NaCl, 20 mM NaH_2PO_4 (pH 7.4), 2 mM EDTA (pH 7.4)) for 5 min before being allowed to dry at room temperature for 30-60 min. Dry filters were then baked between two sheets of dry 3 MM paper at 80°C for 2 h.

Screening of phage plaques with radioactive probes. (Benton and Davies, 1977).

This method was used to screen large numbers of recombinant phage plaques. After overnight incubation at 37°C of large (20x20 cm, Nunc) Petri dishes with 90% confluent plaques were incubated for 1 hour at 4°C. Nitrocellulose filters (Schleicher and Schüll) were layered onto the plates and allowed to stand for 30 seconds to 1 min (replica filters were left longer, 1-3 min) before being carefully peeled off and layered onto 3 MM paper soaked with 0.5 M NaOH. After 5 min the filter was transferred to a second sheet of 3 MM paper soaked in 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl. Finally the filter was washed in 2x SSC and then allowed to dry at room temperature before being baked at 80°C for 2 h. Filters were hybridised as described for Grunstein and Hogness blots.

Hybridisation of DNA bound to nitrocellulose filters.

This method was used both for hybridisation of DNA bound to nitrocellulose filters from colony and plaque lifts.

Baked filters were prehybridised in 10-20 ml of a buffer containing 0.2% polyvinyl-pyrrolidone (MW 60,000), 0.2% Ficoll (MW 400,000), 0.2%

bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 0.1% Na-pyrophosphate, 0.5% SDS and 100 µg/ml of denatured herring sperm DNA for 10-16 h at 68°C with constant shaking in a sealable plastic bag.

After prehybridisation the buffer was removed and replaced with an equal volume containing 1-2 x 10^6 dpm/ml of denatured radioactive probe. The plastic bag was resealed and incubated for a further 12-14 h at 68°C with constant agitation. Filters were washed twice in 100 ml of 2x SSC at room temperature for 5 min, then twice in 200 ml of 0.1x SSC, 0.1% SDS at 68°C for 30 min and finally once in 100 ml 0.1x SSC, 1% SDS for 20 min at room temperature, before being blotted between two paper towels and exposed to X-ray film at -70°C.

Labelling of cells with ^{35}S-methionine.

Log phase cells were washed once in PBS before being either resuspended (for non-adherent cells) or fed with GMEM containing 10% FCS (this medium contained 1/10 the normal concentration of methionine). Cells were incubated for 6 h in this medium before being washed twice in Earle's buffered saline. Cells were labelled for 1 hourin GMEM containing 10% FCS without methionine and 25 µCi/ml ^{35}S methionine.

After this incubation period, the medium was removed and the cells were washed twice in ice cold PBS. Cells were either pelleted and resuspended in 100 µl 10 mM Tris (pH 7.2) or scraped off and resuspended in 100 µl of the above buffer. Samples were either stored at -70°C or used immediately for immune precipitation.

Immune precipitation. (P. Nobis, pers. comm., University of Hamburg)

Preparation of extract.

Cells were lysed in 1 ml of extraction buffer (5 mM Tris (pH 9.2), 1 mM EDTA, 400 mM KCl, 1% Triton X-100, 1 mM PMSF, added just before use from a 100 mM stock solution in DMSO) by mixing on a vortex for 1 min before incubating on ice for 15-30 min. The lysed cells were then centrifuged for 15-20 min in an Eppendorf centrifuge. The supernatant was removed to a fresh tube ready for precipitation.

Preparation of a Staphylococcus aureus reagent (cell adsorbed).

S.aureus were prepared as described by Kessler (1975) and was stored at -20°C in aliquots in PBS-azide. Before use S.aureus was pelleted in an Eppendorf centrifuge (1-2 min) and washed once in 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 0.5% NP40 (TEN containing 0.5% NP40) and then resuspended in the same buffer. Preadsorbed S.aureus were prepared as follows. An unlabelled cell lysate was prepared as above and the S.aureus were diluted 1:10 in this lysate and incubated for 1 hour on ice. The S.aureus were then pelleted for 2 min in an Eppendorf centrifuge and then washed 2-3 times in TEN containing 0.5% NP40, before being resuspended in the start volume of TEN containing 0.5% NP40.

Precipitation.

0.4 - 1 ml of the labelled extract was added to calf serum-treated Eppendorf tubes (incubated with PBS containing 10% FCS at 37°C for 1 hour, washed twice in PBS, aspirated dry) together with 10 µl of a normal serum (either goat or mouse, depending on the antiserum to be used) for 15-20 min on ice. After this period 60 µl of S.aureus were

added and incubated on ice for 15-20 min. S.aureus was pelleted by centrifugation for 2 min in an Eppendorf centrifuge at 4°C. The supernatant was pipetted into a fresh tube and incubated with 10-50 µl of antiserum (depending on the source) and incubated for 15-30 min on ice. After addition of 60 µl of S.aureus and incubation on ice for 30 min, the S.aureus antibody-antigen complex was pelleted in an Eppendorf centrifuge for 2 min at 4°C. The antibody specific pellet was then washed three times in TEN containing 0.5% NP40 and once in TEN containing 0.5% NP40 and 2.5 M KCl. The resulting pellet was resuspended in 30 µl of cracking buffer (10 mM Tris-HCl (pH 7.2), 10% SDS, 25% 2-mercaptoethanol, 25% glycerol, 0.1% bromophenol blue) and boiled for 5 min before being analysed by SDS-PAGE.

Preparation of gels for fluorography. (Bonner and Laskey, 1974).

After electrophoresis the gel was placed in DMSO and gently shaken for 30 min at room temperature. The DMSO was replaced with fresh DMSO and incubated as above. The gel was then transferred to 4 volumes of 22.2% PPO (w/v) in DMSO and incubated with gentle shaking for 3 h at room temperature. The gel was soaked in running water for 15-20 min to swell the gel and remove DMSO, and also to precipitate the PPO. Afterwards the gel was dried under vacuum at 80°C in a Bio-Rad gel dryer for 2 h. Dried gels were exposed to preflashed Kodak X-omat H film at -70°C.

Electrophoretic transfer of proteins (Western blotting) to nylon nitrocellulose filters: Gene Screen plus.

After electrophoresis the gel was equilibrated for 30-60 min in 25 mM Tris-HCl (pH 8.3) and 192 mM glycine. Membrane cut to the exact size of

the equilibrated gel was soaked in the above buffer for 15-20 min together with two Scotch-Brite pads and two pieces of Whatman 3 MM filter paper. A sandwich was prepared as follows. One piece of filter paper was placed onto a Scotch-Brite pad, onto which was then placed the equilibrated gel. The gene screen was then placed onto the gel, avoiding the trapping of air bubbles. The second piece of 3 MM paper was placed onto the filter and the sandwich was completed with the second Scotch-Brite pad. The sandwich was then inserted into a transfer apparatus so that the gene screen membrane was positioned between the positive electrode and the gel. Transfer was performed in 25 mM Tris and 192 mM glycine at 30 V (10-15 mA) overnight (16 h) at 4°C.

Immunological detection of proteins transferred to Gene Screen plus membranes.

After transfer, the membrane was carefully removed from the gel and allowed to dry for 30 min at room temperature. The membrane was then incubated in an excess of blocking solution (100-200 ml, 10% Instant Non-Fat dry milk in PBS) for 1 hour at room temperature. The blocking solution was removed and the filter incubated in 10 ml of incubation solution (10% Instant Non-Fat dry milk in PBS containing 0.03% Tween 20 with 100-200 µl of antiserum) in a sealed plastic bag at room temperature for 1 hour with constant agitation. The membrane was then washed three times in an excess of washing solution (PBS containing 0.03% Tween 20) for 5 min. After washing, the membrane was incubated in 10 ml of incubation solution containing anti-serum IgG peroxidase conjugated antibody (1:1000 dilution) in a sealed plastic bag for 2 h at room temperature with constant agitation. The membrane was then washed three times 10 min each in an excess of washing solution and

three times 2 min each in distilled water. The gel was then stained for 5-15 min in the following mixture of solution: 2ml chloronaphthol (3mg/ml) in methanol, 10 ml 200 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), 30 µl H_2O_2, and for 1-2 min in H_2O to intensify the staining. Membranes were dried between two sheets of 3 MM paper and stored at room temperature in the dark.

Neomycinphosphotransferase II (NPT II) assay. (C. Stewart, pers. comm., EMBL, Heidelberg).

In summary, the proteins of a cell extract are separated on a non-denaturing polyacrylamide gel. The position and amount of enzyme in the gel is determined by phosphorylation of the antibiotic Kanamycin with a radiolabelled ^{32}P molecule. For this the substrates, Kanamycin and γ-^{32}P ATP, are fixed in an agarose gel which is then placed onto the protein gel. After the enzyme reaction the radiolabelled Kanamycin is transferred and fixed onto P81 ion exchange paper and monitored by autoradiography.

Protein extracts (20 µl, 5 x 10^6 cells) from either organs (spleens) or cells sonicated in TE (pH 8.0) were mixed with an equal volume of 2x sample buffer (0.2% SDS, 125 mM Tris (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue). The samples were then separated on a 10% non-denaturing acrylamide gel (10% acrylamide, 0.33% bis-acrylamide, 0.35 M Tris-HCl (pH 8.8)) at 50 V for 12-16 h.

The gel was washed twice for 5 min in water and equilibrated for 30 min in 67 mM Tris-maleate, 42 mM $MgCl_2$, 400 mM NH_4Cl (pH 7.1). The gel was transferred to a glass plate before being covered with a 1.5 mm thick 1% agarose gel containing 20 µg/ml Kanamycin sulphate, ca. 3 mM γ-^{32}P ATP, and incubated at room temperature for 30 min.

The gel sandwich was covered with one sheet of Whatman P81 paper, two sheets of Whatman 3 MM paper and a stack of paper towels. Transfer was continued for 3 h. The P81 paper was transferred to a sealable plastic bag containing 10 mM Na_2PO_4 (pH 7.4), 0.1% SDS, and 1 mg/ml proteinase K, incubated at 60°C for 30 min, and washed three times in cold water for 5 min, twice in hot water (80°C) for 10 min before being washed again twice (5 min) in cold water. Finally the P81 paper was washed in hot (80°C) 50 mM Na_2PO_4 (pH 7.4) for 5 min before drying briefly at room temperature. The paper was then exposed with an X-ray film at -70°C.

DNA sequencing:

Cloning of DNA fragments into M13 mp series vectors was essentially as for subcloning into plasmids, except where otherwise specified.

M13 single stranded phage DNA.

This method was employed whenever M13 single stranded DNA was required for the chain termination method of DNA sequencing. All M13 strains used were of the mp series constructed by Messing (Messing, 1979) and contained a piece of the Lac region encoding a peptide fragment of β-galactosidase capable of α-complementation with the partial product encoded by the F Episome present in the JM101 host strain (Messing and Gronneborn, 1978; Gronneborn and Messing, 1978). The status of the M13 Lac region, functional or otherwise was indicated by plating out phage in H-Top agar containing the Lac inducer IPTG (200 µg/ml) and the chromogenic substrate BCIG (x-gal) 200 µg/ml onto minimal agar plates (1.05% K_2HPO_4, 4.5% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.5% Na-citrate.H_2O, 0.02% $MgSO_4 \cdot 7\ H_2O$, 0.2% glucose, 5 mg/l Vitamin B1,

1.5% agar). A single plaque of the appropriate colour was picked with a sterile tooth-pick and grown for 8 h at 37°C in 2 ml of 2x YT medium (0.5% NaCl, 1% yeast extract, 1.6% tryptone), with vigorous shaking. The host bacteria were pelleted by centrifugation for 5 min in an Eppendorf centrifuge. 800 µl was carefully transferred to a fresh tube. To this was then added 200 µl 2.5 M NaCl, 20% PEG 6000 and after thorough mixing was incubated at room temperature for 15 min. The precipitated phage was pelleted by centrifugation for 10 min in an Eppendorf centrifuge. All traces of the supernatant were aspirated and the pellet was resuspended in 100 µl TE_x (TE sequencing 10 mM Tris (pH 8.5), 0.1 mM EDTA). Protein was removed by a single equilibrated phenol extraction (50 µl). After centrifugation for 2 min in a microfuge the DNA in the aqueous phase was precipitated by addition of 10 µl 3M Na-acetate (pH 5.2) and 250 µl ice cold ethanol and incubation at -20°C for 2 h to overnight.

DNA sequencing reactions.

The procedure adopted was essentially that of Sanger et al. (1977) with minor modifications (Gibco/BRL, M13 cloning/dideoxy sequencing: Instruction Manual 1985).

For each clone sequenced, approximately 0.5-1 µg single stranded DNA in 3-5 µl H_2O was mixed with 2 µl (4 ng) of M13 17-base sequencing primer, 1 µl of 10x polymerase reaction buffer (70 mM Tris-HCl (pH 7.5), 7.0 mM $MgCl_2$, 500 mM NaCl), and H_2O to give a final volume of 12.4 µl. The components were then mixed and centrifuged briefly to recover the contents at the bottom of the tube. DNA was denatured by boiling for 5 min (100°C) before being allowed to equilibrate to room temperature over a period of 30-45 min. This step allows annealing of

the primer to the template. This primer/template preparation was either stored on ice or at -20°C until the sequencing reactions were ready.

During the primer annealing reaction the following was added to the sides of four reaction mix centrifuge tubes in separate steps:

Tube A: 1 µl A° mix, 1 µl diluted ddA (0.5 mM)

Tube C: 1 µl C° mix, 1 µl diluted ddC (0.5 mM)

Tube G: 1 µl G° mix, 1 µl diluted ddG (0.5 mM)

Tube T: 1 µl T° mix, 1 µl diluted ddT (1.0 mM).

Mixtures were prepared as follows (stored at -20°C):

	A°	C°	G°	T°
0.5 mM dATP	1 µl	20 µl	20 µl	20 µl
0.5 mM dGTP	20 µl	20 µl	1 µl	20 µl
0.5 mM dTTP	20 µl	20 µl	20 µl	1 µl
10x polymer neutr.buffer	20 µl	20 µl	20 µl	20 µl.

After temperature equilibration, the following was added directly to the tube containing the hydrolysed primer/template:

1 µl (α-^{32}P) dCTP (aqueous solution, 400 Ci/mM),

1 µl 0.1 M DTT,

1 µl (1 unit/µl) large fragment DNA polymerase I (Klenow fragment).

The contents were gently mixed and 3 µl aliquots were pipetted into the four A, C, G and T tubes. The reactants were mixed and collected by centrifugation to form a single drop and incubated for 20 min at 50°C. After this time 1 µl of 0.5 M dCTP was added to each of the reaction tubes and incubated for an additional 10 min at 50°C. The reactions were then dried under vacuum before being resuspended in 4 µl of stop buffer (0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM Na_2-EDTA, 95% deionized formamide). The reactions were then denatured by boiling for

5 min at 90-100°C and 2 µl was loaded onto a 0.4 mm thick 6% acrylamide (19:1 acrylamide:bis-acrylamide) gel containing 7 M urea, run in 1x TBE buffer. The gel was run at a constant power of approximated 50 W or at a voltage judged to give a running temperature of approximately 45°C, with the use of aluminium plates. The gel was removed and exposed to X-ray film without the use of intensifying screens at -70°C.

Results

Chapter 1

Molecular analysis of FV extrachromosomal DNAs found in Friend cells

Introduction

The malignant stage of the Friend disease results in the formation of a transformed population of cells that can be established in vitro (Friend et al., 1966; Ostertag et al., 1972; Ikawa et al., 1973) and induced to terminally differentiate along the erythroid pathway by addition of a variety of chemical agents to the culture medium (Friend et al., 1971; Reuben et al., 1980). Various studies questioning viral involvement in the establishment and maintenance of the transformed state have utilized various biological and biochemical assays to examine the Friend virus complex in murine erythroleukaemia cells (Friend leukaemia cells, FLC) that have been exposed to the differentiation inducing agent DMSO. One of the earliest findings was an increase in the number of budding virus particles on the surface of DMSO treated Friend cells as detected by electron microscopy (Sato et al., 1971). However, it could not be shown in these studies whether or not the increase in the number of virus particles represented an increased synthesis of virus or an inhibition of virus release. Additional work indicated that this commonly observed phenomenon that occurs in many separately established cell lines is a transient increase in virus synthesis after exposure to DMSO (Dube et al., 1975; Ikawa et al., 1976; Ostertag and Pragnell, 1978; Ostertag et al., 1979; Fagg et al., 1980). Increases in SFFV activity (from 5-200 fold) has been reported during the first two days of DMSO treatment, coupled together with a change in the tropism of the helper virus, thus suggesting that DMSO treatment also causes the induction of an endogenous virus in Friend cells (Dube et al., 1975; Ostertag and Pragnell, 1978; Ostertag et al., 1979).

Variant cell lines also exist that show no increase in viral synthesis, although they may still be induced to differentiate. Thus from these and various other observations it has been suggested that the augmentation of virus gene expression and the induction of differentiation are independent events (Dube et al., 1975; Ikawa et al., 1976; Sherton et al., 1976; Tsuei et al., 1979). Sherton et al. (1976) suggested that this increase in virus synthesis may be related to the effects of DMSO on the cellular growth cycle of Friend cells. Recent experiments using a retrovirus vector containing a complete human β-globin gene showed that, after infection and selection of FLCs, the β-globin gene was inducibly expressed after addition of DMSO (Cone et al., 1987).

It has also been observed that an extrachromosomal SFFV DNA was present in one DMSO resistant Friend cell line (Ikawa et al., 1980). A deeper analysis of this aspect (Kern and Axelrod, 1983) revealed that extrachromosomal DNAs of SFFV (both circular and linear) found in the cytoplasm of the examined Friend cell lines were inducible with, and were indeed dependent upon, the continued presence of DMSO. They demonstrated that a greater than eight fold variation was observed in the amount of this DNA, with the largest amounts being found in those cells that were resistant to the induction of differentiation by DMSO.

Based on these observations it was decided to examine various Friend cell lines, made using the Mirand strain of F-SFFV (Mirand, 1967), which have been isolated and previously described by Ostertag and co-workers (Eisen et al., 1978; Ostertag et al., 1979). The studies of Kern and Axelrod (1983) used Friend cells (the original cell line 745A and siblings there of) made with an original isolate of the Friend

virus complex from C. Friend.

Results

The cell lines used in this study and their origin are depicted in Table 2. The cell lines F4N, F4-6 (F4-6 being a clone of the original Friend cell line F4N) and B8/3 represent cells which may be fully induced to terminally differentiate after the addition of DMSO, (Ostertag and Pragnell, 1981). B8/3 differs from the other two cell lines in that, due to a defect in helper virus function in which pr67 gag is synthesized but is not aberrant and not shed, there is no constitutive release of F-MuLV and SFFV and very little or no DMSO-induced release of SFFV.

F4N+2 and F4+ may only be induced by DMSO to proceed to an early phase of differentiation (a precommitment phase) in which spectrin synthesis is induced but progression to a committed stage, in which haemoglobin is synthesized, is blocked. There is no constitutive virus release in F4N+2 although F4+ release high titres of SFFV. Both cell lines show no DMSO-induced virus release.

a) Optimisation of the conditions for DMSO induction of the Friend cell lines F4N, F4-6, B8/3, F4N+2 and F4+

Although published work from this and other laboratories have already defined the optimal DMSO-induction conditions, for these cells, it was decided to re-optimise the DMSO induction conditions as inducibility may vary with the medium and/or serum source. Also all cells used for this and further studies were from a single batch of cells, frozen and stored in liquid nitrogen at the start of this work in order to ensure

Table 2. Properties of the mouse erythroleukaemia cells (Friend cells) used in the analysis of extrachromosomal DNA species.

cell line*	differentiation inducibility	constituitive virus release	DMSO induced virus release
F4N	full	high	high (SFFV)
F4-6	full	high	high (SFFV)
B8/1	full	none	none
F4N+2	early phase of differentiation (spectrin)	none	none
F4+	none	high (SFFV)	none

*The origin of these cell lines is described in Materials and Methods.

uniformity.

An exhaustive description of the induction protocol is shown in Figure 13.

Titration results for all cell lines are depicted in Figure 14. From these results it can be seen that using the protocol described, an optimal induction (as determined by benzidine staining) for all cells, except the non-inducible cell line F4+, is obtained at a concentration of 1.5% DMSO, whereas suboptimal degrees of differentiation were obtained when using higher or lower concentrations. These results obtained here correlate well with those previously published (Eisen et al., 1978; Ostertag et al., 1979).

b) Subcloning of specific hybridisation probes

A prerequisite for the planned study was to be able to identify and discriminate between F-MuLV and F-SFFV-specific sequences. Although very different in size, the two viruses are closely related and share a large proportion of their nucleotide sequences, (see introduction) including LTR, gag and pol sequences, although the latter two genes are altered by deletions in SFFV. However, one region which specifically differs between the two is the 5' env region, where the sequences in SFFV are highly related to MCF viruses and not to F-MuLV. This information was used to subclone specific nucleotide sequences which could be used as hybridisation probes, the strategy of which is shown in Figure 15.

Using available restriction enzyme and nucleotide sequence data, the following two env (and thus virus specific) probes were subcloned. From a molecularly cloned F-MuLV clone (clone 57, Oliff et al., 1980) obtained from S. K. Ruscetti (NIH Bethesda, Washington) a 700 bp 5' env

Figure 13. DMSO-induction protocol for Friend Cells

1) Cells grown in log phase in GMEM + 10% FCS at 37°C in 5% CO_2 air for 3-4 days before induction.
2) Dilute log phase cells (approximately 10^6/ml) 1:100 in fresh growth medium, GMEM + 10% FCS. Incubate for 48 h at 37°C, 5% CO_2 in air. (Concentration is then about 5 x 10^8 cells/ml.)
3) Remove 50% of the culture and replace with GMEM + 10% FCS + DMSO (double the end concentration needed, usually 1-1.5% end concentration).
   This is designated day 0. Incubate at 37°C, 5% CO_2 in air.
4) Incubate for 48 h, then replace 50-75% of the culture with GMEM + 10% FCS + DMSO (day 2).
5) Incubate for a further 72 h at 37°C, 5% CO_2 in air. The cell density is then approximately 10^6/ml, and the pH of the medium is slightly alkaline. This is designated day 5, and the percentage of cells synthesizing haemoglobin is determined by benzidine staining.

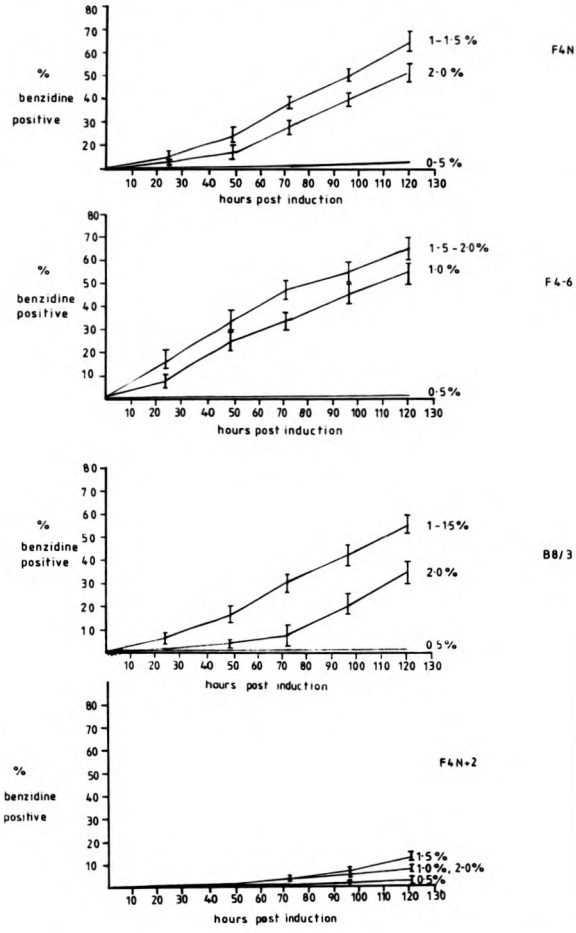

F4N
1-1·5 %
2·0 %
0·5 %
% benzidine positive
hours post induction
F4-6
1·5 - 2·0%
1·0 %
0·5%
B8/3
1-15%
2·0%
0·5%
F4N+2
1·5 %
1·0%, 2·0%
0·5%

Figure 14. Titration of DMSO concentrations needed for optimal induction of the Friend cells used in this study.

The Friend cell lines F4N, F4-6, F4N+2, B8/1 and F4+ were incubated with various concentrations of DMSO (0.5%-2%) as described in Figure 13.

At 24 hour intervals an aliquot of cells was removed from the culture and stained with benzidine as described in Materials and Methods. The results for the cell line F4+ are not shown, for at no DMSO concentration could the cells be induced to differentiate to give benzidine positive cells (above a very low background of approximately 0.5% positive cells).

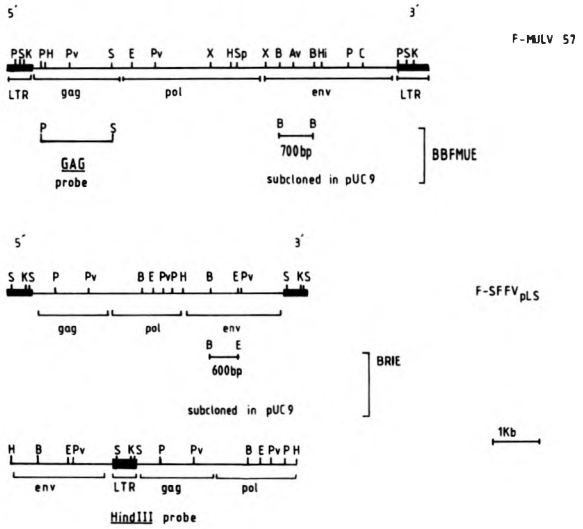

5'
3'
F-MULV 57
PSK PH Pv S E Pv X HSp X B Av BHi P C PSK
LTR gag pol env LTR
P S
GAG
probe
B B
700bp
subcloned in pUC 9
BBFMUE
5'
3'
S KS P Pv B E PvPH B EPv S KS
F-SFFV pLS
gag pol env
B E
600bp
BRIE
subcloned in pUC 9
1Kb
H B EPv S KS P Pv B E PvPH
env LTR gag pol
HindIII probe

Figure 15. Subcloning of env-specific sequences for use as molecular hybridisation probes

Shown is the strategy used to subclone, env specific probes, which after labelling with ^{32}P could be used to differentiate between F-MuLV and F-SFFV specific sequences. The origin of the starting plasmids for the cloned F-MuLV and F-SFFV sequences are described in the text.

The restriction enzyme lettering code is as follows, P, PstI; S, SstI: K, KpnI: Pv, PvuII: E, EcoRI: X, XbaI: H, HindIII: Sp, SpHI: B, BamHI: Av, AvaII: Hi, HincII: C, ClaI.

The maps depicted are drawn to scale, and are based on published data cited in the corresponding text.

Also shown is the permutated HindIII fragment representing the whole of the SFFVp genome and referred to in the text as the HindIII probe. The origin of a restriction enzyme fragment used as a gag specific probe is also illustrated (GAG).

specific BamH1 fragment was isolated from a preparative gel and cloned into the BamH1 site of the pUC9 cloning vector. The resulting plasmid was named pBBFMUE. Identical procedures were used to subclone a 600 bp BamH1-EcoR1 5' env fragment from a molecularly cloned polycythaemia strain of F-SFFV (pSFFVp, Linemeyer et al., 1980) to yield a plasmid called pBRIE.

That these plasmids, or the subcloned fragments therein, hybridised specifically to their respective viruses was shown in the following experiment. 100 ng of the plasmids pF-MuLV clone 57, pSFFVp, and the cloning vector pUC9, in 10 µl TE solution, were denatured by boiling for 5 min before being spotted onto nitrocellulose paper (BA85, Schleicher and Schüll). These dot blots were then hybridised under stringent conditions (as in Materials and Methods) with ^{32}P radiolabelled env fragments isolated from the plasmids pBBFMUE and pBRIE, the results of which are shown in Figure 16.

From these results it can be seen that the 700 bp pBBFMUE fragment (Figure 16a) only hybridises to F-MuLV and the 600 bp pBRIE fragment only hybridises to F-SFFV under these conditions (Figure 16b).

A further probe, a 6.3 kb HindIII fragment (present as a permutated linear molecule in pBR322) comprising the whole of F-SFFV genome from the pSFFVp plasmid hybridised to both the F-MuLV and F-SFFV sequence (Figure 16c) thus showing the nucleotide similarity of these viral genomes. No hybridisation to the cloning vector was seen.

c) Molecular analysis of extrachromosomal DNA found in Friend cells

The presence of extrachromosomal viral DNA during the course of DMSO-induced differentiation was investigated using the cells, induction protocol and specific probes described above. Low molecular

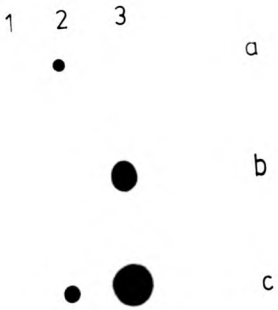

1
2
3
a
b
c

Figure 16. Specificity of the subcloned fragments, BRIE and BBFMUE

The specifity of the subcloned BRIE and BBFMUE fragments was tested by dot blot analysis.

Isolated BRIE and BBFMUE fragments (free of the pUC9 vector, after restriction enzyme digestion, and isolation by electroelution) were labelled with ^{32}P, by the nick translation method described in Materials and Methods, and bybridised under stringent conditions, with the nitrocellulose bound plasmids, pF-MuLV, pSFFVp and pUC9.

1, 2 and 3 represent the plasmids pUC9, pSFFVp and pF-MuLV respectively.

The probes used were a: BRIE,

b: BBFMUE,

c: a HindIII fragment comprising the whole of the SFFVp genome.

weight DNA, free from contaminating high molecular weight DNA, was extracted by the method of Hirt (1967).

In this series of experiments, the cells were induced to differentiate according to the protocol described in Figure 13. At intervals of 24 h 5 x 10^6 cells were removed from 20 ml cultures and Hirt supernatants were prepared. After a 30 min RNAse digestion to remove residual contaminating RNA, the DNA samples were electrophoresed on 0.8% agarose gels containing 0.5 µg/ml EtBr before being transferred to Gene Screen by the method of Southern (1979). Blots were then hybridised with respective ^{32}P labelled DNA probes, the results of which are shown in Figures 17, 18, 19.

The results shown in Figure 17 represent those obtained with the cell line F4-6, and the HindIII pSFFVp probe, which hybridises with both SFFV and F-MuLV DNA sequences. Also shown are the percentage of benzidine positive cells (B^+) at the various sampling times post induction and a UV transilluminator polaroid of the gel before it was blotted. This acted as an internal control to show that the Hirt supernatant procedure had worked to similar degrees of efficiency, for one always has a small percentage of contaminating high molecular weight DNA, which because the DNA is not digested with restriction enzymes stays at the top of the gel.

It can be seen that at day 0, when only 1-2% of the cells are benzidine positive, various bands are visible (highlighted by arrows), one at roughly 9 kb, a faint band at approximately 7 kb and one at 3.5 kb. These sizes would suggest (due to the nature with which DNA molecules with differing conformations migrate in the presence of EtBr) that they correspond to a linear DNA of F-MuLV (at 9 kb), a linear DNA of SFFV (at 7 kb) and to a circular DNA of one of the above viruses

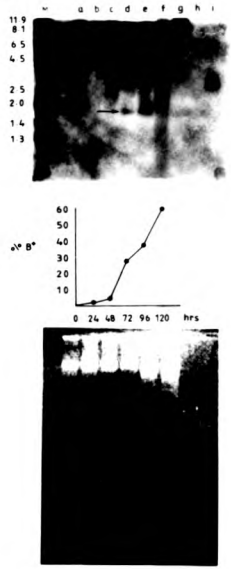

M a b c d e f g h i
11.9
8.1
6.5
4.5
2.5
2.0
1.4
1.3
60
50
40
30
20
10
% B*
0 24 48 72 96 120 hrs

Figure 17. FV extrachromosomal DNA present in the Friend cell line F4-6

Southern blot analysis of Hirt supernatants extracted from cells at 24 hour intervals following DMSO induction (p.d.i.). The probe used in this analysis was the HindIII fragment wich detects both F-MuLV and SFFV sequences.

Lane a: 0 h p.d.i.,
b: 24 h p.d.i.,
c: 48 h p.d.i.,
d: 72 h p.d.i.,
e: 96 h p.d.i.,
f: 120 h p.d.i.,
g: supercoiled 12 kb marker plasmid,
h: supercoiled 9 kb marker plasmid,
i: supercoiled 6 kb marker plasmid.

The numbers to the left represent molecular weight markers in kb. Also shown (middle) are the kinetics of induction of benzidine positive (B^+) cells and (bottom) a polaroid of the agarose gel before blotting. The contaminating, high molecular weight DNA acted as an internal control.

The samples were run for 16 h in a 0.8% agarose gel in TAE buffer in the presence of 0.5 µg/ml EtBr.

although the position at which it runs would suggest that it corresponds to a SFFV-related molecule. After 24 h and 48 h respectively the bands seen on day 0 were still present although appreciably changed in their intensity (although this is a long exposure the bands are not clearly visible). However, after 72 h post induction when approximately 30% of the cells were benzidine positive two new bands appeared, which ran slower than the 3.5 kb band at approximately 4.0 kb and notably further at approximately 2 kb. These bands persisted and were detectable at 96 h, but were again not detectable at 120 h post induction when 60% of the cells were benzidine positive. The polaroid demonstrates that the amount of contaminating genomic DNA is roughly equal in all samples thus suggesting that there was no great discrepancy in the amount of sample being loaded, which could have led to those results. From such results one could thus infer that DNA molecules corresponding to bands at 4 kb and 2 kb were being induced during the differentiation process of the cells, and reached a detectable peak at 72-96 h post induction before being again not detectable at 120 h post induction. This observation was seen in numerous experiments. A similar finding was also seen with the cell line F4N, but as F4-6 is indeed a subclone of this cell line and is biologically indistinguishable from F4N then this is not surprising.

To reveal the identity of the bands described above the blot was washed free of bound HindIII pSFFVp probe and rehybridised with the F-SFFV env specific ^{32}P-labelled pBRIE probe. However due to high background problems no discrete bands could be detected that could be reproduced as a figure, although later analysis was able to identify unequivocaly the specificity of the bands, see large scale preparation.

Thus it appears that in this cell line there is an induction of

Friend virus related extrachromosomal DNA molecules. From size and the behaviour of the various DNA molecule forms in agarose gels containing EtBr one could suggest that these molecules correspond to circular equivalents of F-SFFV (which is also suggested by the migration of supercoiled plasmid controls seen on all gels) although no unequivocal proof can be presented at the moment. On examining the partially inducible cell line F4N+2 in exactly the same manner (Figure 18) then one encounters a similar picture in which F-SFFV specific DNA molecules are induced at a peak of 72 h post induction, although possibly to not such a great extent as F4-6. Using the HindIII pSFFVp probe and comparing with the results obtained for F4-6 one can identify the bands as putative linear F-MuLV, linear F-SFFV and circular F-SFFV molecules, as well as the unidentified bands seen above. Analysis of the non-inducible cell line F4+ (Figure 19) showed no induction of any viral extrachromosomal DNAs throughout the whole 120 hours, although F-MuLV related viral DNAs are indeed present. These cells are refractory to the effects of DMSO, with a background level of <1% benzidine positive cells after 120 h post induction. Repeated analysis of the cell line B8/1 was unable to detect any extrachromosomal DNAs whatsoever.

These results thus tend to suggest that there may be a correlation between the inducibility of the cell and the induction of SFFV viral extrachromosomal DNAs. One interesting fact however, is that no extrachromosomal DNA of any detectable origin was detectable in the cell line B8/1, which has a helper virus defect (a gag gene defect) which may be interpreted to mean that, a replication competent F-MuLV (or even more specifically a functional gag gene product) could be somehow involved in this phenomenon. These results, however, do not correlate with the inducibility of virus release, for as shown in

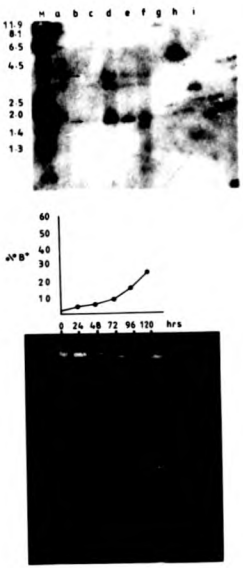

M a b c d e f g h i
11.9
8.1
6.5
4.5
2.5
2.0
1.4
1.3
60
50
40
30
20
10
0 24 48 72 96 120 hrs

Figure 18. FV extrachromosomal DNA present in the Friend cell line F4N+2

Figure legend as for Figure 17.

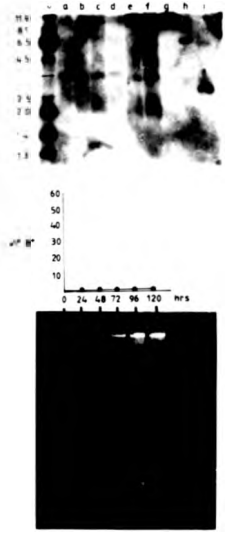

a b c d e f g h i
60
50
40
30
20
10
0 24 48 72 96 120 hrs

Figure 19. FV extrachromosomal DNA present in the Friend cell line F4+

Figure legend as for Figure 17.

Table 2, although F4N+2 is a partially inducible cell line, it neither constitutively releases virus nor is there any DMSO-induced virus release, although extrachromosomal DNAs were indeed induced. However, the cell line F4+ which is non-inducible and indeed shows no differentiation inducible extrachromosomal DNAs, does in fact release high levels of SFFV constitutively, although none is induced after DMSO induction. This contradicts the results of Kern and Axelrod (1983).

d) Subcellular location of extrachromosomal DNA

From the results shown it can be seen that all cells analysed apart from 88/3 contained extrachromosomal viral DNA, although to varying extents and specificities. To further analyse this and also aid in subsequent large scale preparation and purification of these DNAs the intracellular (nuclear or cytoplasmic) location of these extrachromosomal DNAs was determined.

The cell line used for this analysis, F4-6, was grown at log phase conditions in GMEM + 10% FCS without the presence of DMSO. The cells were then induced to differentiate as described above and after 3 days nuclear and cytoplasmic fractions were prepared and analysed for the presence of extrachromosomal DNAs by agarose gel electrophoresis (in the presence of EtBr) Southern blotting and subsequent nucleic acid hybridisation. The results of this analysis are shown in Figure 20. From these experiments it is clear that the extrachromosomal DNAs seen in these cells are present within the cytoplasm of these cells as in the studies of Kern and Axelrod (1983). Indeed as in these experiments the BRIE probe was used as a hybridisation probe, the DNA molecules were identified to be of SFFV related origin, i.e. a 7 kb linear molecule, a closed circular molecule (which ran at 4 kb) and possibly a


M 1 2 3 4 5 6 7 8 9
11.9 -
8.1 -
6.5 -
- genomic
- linear
- circular

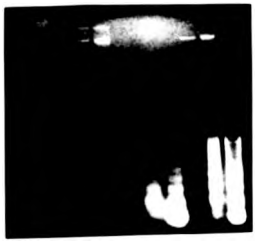


Figure 20. Cellular location of the FV extrachromosomal DNA found in the cell line F4-6

Southern blot analysis of Hirt supernatants from subcellular fractions (nuclear and cytoplasmic) of DMSO-induced F4-6 cells 72 h p.d.i.

Nuclear fraction:

Lane 1: 5 x 10^8 cells.

Lane 2: 1 x 10^8 cells.

Lane 3: 1 x 10^7 cells.

Cytoplasmic fraction:

Lane 4: 5 x 10^8 cells.

Lane 5: 1 x 10^8 cells.

Lane 6: 1 x 10^7 cells.

Unfractionated Hirt supernatant:

Lane 7: 5 x 10^8 cells.

Lane 8: 1 x 10^8 cells.

Lane 9: 1 x 10^7 cells.

For this analysis the pBRIE fragment was used as a hybridisation probe. The number to the left represent molecular weight markers in kb. Also shown is a polaroid of the EtBr stained gel before Southern blotting.
The DNA samples were electrophoresed in a 0.8% agarose gel in TAE buffer for 12 h in the presence of 0.5 µg/ml EtBr.

supercoiled molecule or related variant that ran at approximately 2.5 kb. No extrachromosomal viral DNA was detectable in the nucleus.

e) Absence of extrachromosomal DNAs in non-producer cells

The presence of retroviral extrachromosomal DNAs in cells is not unique to the observations described above, or those published by Kern and Axelrod (1983). For example unintegrated MMTV DNA is persistently present in non-murine cells infected with the virus. Unintegrated MMTV DNA in infected hepatoma cells is present as linear or open circular duplexes containing two LTRs and also as covalently closed circular viral DNA with either, one or two LTRs (Ringold et al., 1977; Shank et al., 1978).

To analyse the distribution of SFFVp specific extrachromosomal DNAs further it was decided to look in non-erythroid cells (fibroblasts) of both murine and non-murine origin to see if our observations were indeed confined to the system described earlier. For these experiments the SFFVp non-producer and producer fibroblast cell lines depicted in Table 3 were analysed.

Extrachromosomal DNA (Hirt supernatants) from 1 x 10^7 cells was prepared from both stably infected non-producer and producer cell lines either in the presence (1.5%) or absence of DMSO (to simulate conditions subjected to the Friend cells) in the culture medium and the DNA was analysed by agarose gel electrophoresis and Southern blotting. As can be seen from Figure 21, in none of the conditions examined could extrachromosomal DNA of SFFVp or F-MuLV origin (or related origin) be detected, when using a whole SFFV HindIII fragment probe. The figure presented here represents a long exposure. Such results would suggest that the presence of extrachromosomal Friend virus DNA in murine

Table 3. Non-erythroid producer and non-producer fibroblast cell lines,used for the analysis of FV extrachromosomal DNA

Cell Line	Host Cell	Virus*	Reference
SC204+H	SC1 (mouse)	SFFV-P*, F-MuLV*	Bilello et al., 1980
6S21	SC1 (mouse)	SFFV-P*	Bilello et al., 1980
S2D409	SC1 (mouse)	SFFV-P*	Bilello et al., 1980
NRK643/22N	NRK (rat)	F-MuLV*	Bilello et al., 1980

* All SFFV-P were of the Mirand strain.

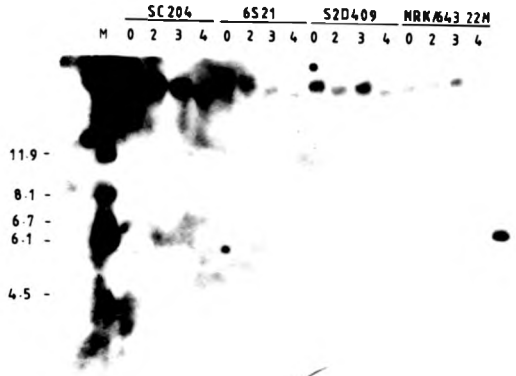

SC204
6S21
S2D409
NRRA643 22N
M 0 2 3 4 0 2 3 4 0 2 3 4 0 2 3 4
11.9 -
8.1 -
6.7 -
6.1 -
4.5 -

Figure 21. Southern blot analysis of Hirt supernatants from producer and non-producer fibroblast cell lines

The origin of the cell lines has been described in Table 3.
0 represents cells that were cultured in the absence of DMSO.
2, 3 and 4 represent cells that were incubated in the presence of 1.5% DMSO, for 48 h, 72 h and 96 h respectively.
The pSFFVp HindIII was used as a hybridisation probe.
The numbers to the left represent molecular weight markers in kb.
The DNA samples were electrophoresed in a 0.8% agarose gel in TAE buffer for 12 h in the presence of 0.5 µg/ml EtBr.

erythroleukaemia cells is possibly restricted to these cells, although examinating a larger selection of cell types would be needed to substantiate this statement.

f) Large scale production of extrachromosomal FV DNA from F4-6

The experiments described so far were all designed to accumulate data as to which erythroleukaemia cell line would be best suited for the isolation and molecular cloning of the extrachromosomal DNA molecules corresponding to the Friend virus complex, F-MuLV and F-SFFVp. Thus from the accumulated data it was decided to use the cell line F4-6, for not only could considerable amounts of these extrachromosomal DNAs be detected in the cytoplasm, but also upon induction, these amounts could be amplified with respect to F-SFFV.

One other interesting observation seen in earlier studies was that not only is an endogenous N-tropic virus induced upon differentiation of the cell line F4N, as compared to the normal NB-tropic virus being released from these cells (Dube et al., 1975), but also that there is a change in genome composition of the Friend virus complex during differentiation of the cell line F4-6 (an observation that could well explain the phenomena seen earlier: Ostertag and Pragnell, 1975). Indeed this induced SFFVp has somewhat different biological properties, as compared to the non-induced virus in that it has a more profound effect on CFU-E formation in the bone marrow of susceptible mice (Fagg et al., 1980).

As a prerequisite for large scale extrachromosomal DNA preparation, F4-6 cells were adapted to growth in roller bottles (in culture volumes of 100ml) over a period of two weeks until the cells could reproducibly be induced to differentiate to give 50-60% benzidine

positive cells after 96 h post induction (as seen in earlier experiments). Such roller bottle adapted cells (5×10^7) were analysed at time intervals post induction with DMSO, as in the previous experiments, for the presence of extrachromosomal viral DNAs. The results of these experiments are shown in Figure 22. The Southern blot depicted in this figure was hybridised with the SFFVp specific BRIE fragment. During the induction and differentiation phase of these cells the amounts of both linear and circular SFFVp DNA increased, thus demonstrating that the roller bottle adapted cells were comparable in this respect to the pre-adapted F4-6 cells used in the previous experiments.

The presence of two circular DNAs which probably represent closed circular molecules with one and two LTRs was also demonstrated in this analysis. It can also be seen that there was no gross detectable alteration in the genome size of SFFVp during the induction, as was reported by Ostertag and Pragnell (1975), although these studies were done at the RNA level.

These results represent somewhat of a paradox as the DNA previously seen to migrate at approximately 2.5 kb (assumed to be supercoiled) was not present. This may be explained by the culture adaptation in roller bottles or equally by the conversion of the supercoiled DNA to a closed circular conformation during the sample preparation.

In order to obtain enough material for restriction enzyme analysis and subsequent molecular cloning, thirty roller bottle cultures were seeded at day 0 with 1×10^8 log phase F4-6 cells/ml (in 100ml) at 37°C, rolled at 2 rpm. After 24 h 100 ml GMEM + 10% FCS + 3% DMSO was added to the culture to give a final DMSO concentration of 1.5%. The cells were then cultured for a further 48 h at 37°C before being fed

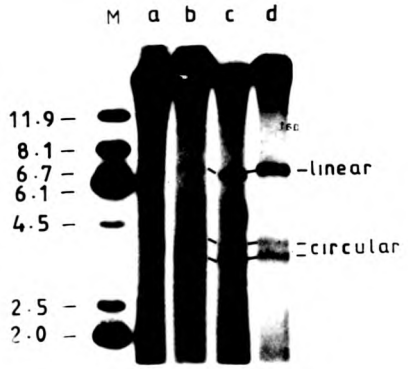

M a b c d
11·9
8·1
6·7
6·1
4·5
2·5
2·0
linear
circular

Figure 22. Southern blot analysis of Hirt supernatants from roller bottle adapted F4-6 cells

DNA samples were electrophoresed in a 0.8% agarose gel in the presence of 0.5 µg/ml EtBr for 12 h before being Southern blotted to a nitrocellulose filter and hybridised with a ^{32}P labelled BRIE probe under stringent conditions. The bands are labelled linear and circular on hand of their migration behaviour in agarose gels in the presence of EtBr.

Lane a: Hirt supernatant from 1 x 10^7 cells at 0 h p.d.i..

Lane b: Hirt supernatant from 1 x 10^7 cells at 24 h p.d.i..

Lane c: Hirt supernatant from 1 x 10^7 cells at 48 h p.d.i..

Lane d: Hirt supernatant from 1 x 10^7 cells at 72 h p.d.i..

The numbers to the left represent molecular weight markers in kb.

with 100 ml GMEM + 10% FCS + 1.5% DMSO. After a further 48 h the cells were harvested (at this time, 120 h post induction 60-70% of the cells were benzidine positive) and extrachromosomal DNA was prepared from the cytoplasm of these cells as in Materials and Methods: From a total of 3.5 x 10^9 cells, 5.25 mg of extrachromosomal DNA was obtained after RNAse digestion and ethanol precipitation. This preparation was then further fractionated on a CsCl gradient (0.94 g/ml) in a 6 ml Beckman quick seal tube in a VT165 rotor for 18 h at 50,000 rpm. After centrifugation 0.5 ml fractions were dialysed over night against 100 fold excess of TE solution. Aliquots of these fractions were then analysed by EtBr agarose gel electrophoresis and Southern blotting. The autoradiograms resulting from these experiments are shown in Figure 23. When the pSFFVp HindIII fragment was used as a hybridisation probe then no detectable signals were seen (Figure 23 A) in the fractions 1, 2 and 3. In fraction 4 there were a number of signals which have been given the nomenclature a-f. These are all extrachromosomal DNAs which have been described previously and can now, in most cases, in conjunction with the BRIE env probe (Figure 23 B), be conclusively identified as, a: contaminating cellular genomic DNA, b: linear F-MuLV, c: linear F-SFFVp, d: circular F-MuLV with one and two LTRs, e: circular F-SFFVp with one and two LTRs and f: which although appears to be F-MuLV related in that it does not hybridise to pBRIE, cannot as yet be identified. Thus it was possible to separate the linear from the circular DNAs (with the exception of band f), although there was still a mixture of F-MuLV and F-SFFVp DNAs.

g) Restriction enzyme mapping of linear F-MuLV and F-SFFVp DNAs

To further analyse the isolated DNAs and thus establish a simple

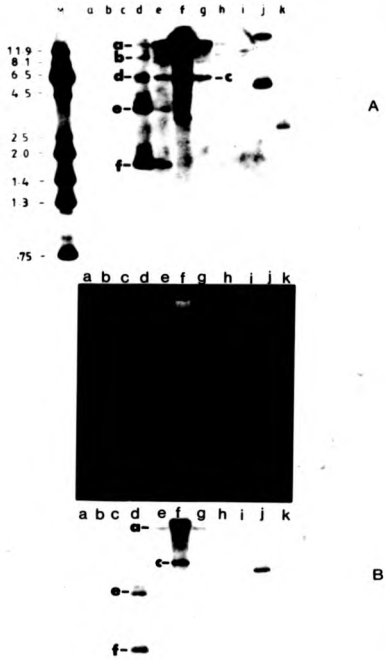

M a b c d e f g h i j k
119
81
65
45
25
20
14
13
.75
a
b
c
d
e
f
A
a b c d e f g h i j k
a b c d e f g h i j k
a
c
e
f
B

Figure 23. Southern blot analysis of CsCl fractionated F4-6 extrachromosomal DNA

Aliquots of the fractionated DNA were electrophoresed in 1% agarose gel at 50 V over night in TAE buffer (0.15 µg/ml EtBr) before being subjected to Southern blot analysis. The blot was hybridised either with the HindIII fragment or with the env specific pBRIE fragment.

Lane a: Fraction 1.

Lane b: Fraction 2.

Lane c: Fraction 3.

Lane d: Fraction 4.

Lane e: Fraction 5.

Lane f: Fraction 6.

Lane g: Fraction 7.

Lane h: Fraction 8.

Lane i: supercoiled 12 kb marker plasmid.

Lane j: supercoiled 9 kb marker plasmid.

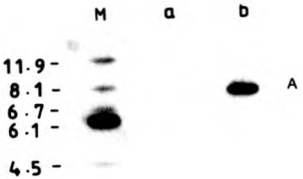
Lane k: supercoiled 6 kb marker plasmid.

The numbers to the left represent molecular weight markers in kb.

restriction enzyme map of F-MuLV and F-SFFVp, the linear DNAs of F-MuLV and F-SFFVp were further purified by preparative agarose gel electrophoresis. For this purpose the fractions 5, 6 and 7 were pooled and run on a 1% preparative agarose gel. Using NA45 paper (described in Materials and Methods) DNA was isolated from regions of the gel, corresponding to the linear F-MuLV (approximately 9.5 kb) and F-SFFVp (approximately 7.0 kb). After dissociation from the NA45 paper and subsequent ethanol precipitation, these isolates were then analysed for the presence of linear DNAs by agarose gel electrophoresis and Southern blotting. The autoradiograph shown in Figure 24 (A and B) shows that both the F-MuLV and the F-SFFVp linear DNAs had been isolated relatively free of contamination (although on longer autoradiograph exposures minor hybridising contaminants could be seen). These DNAs also hybridised to their respective specific *env* probes.

i) *F-MuLV linear DNA.*

Aliquots of the isolated linear DNA were digested with the restriction enzymes *EcoRI*, *XbaI*, *XhoI*, *HindIII*, *SalI*, *SstI* and *SphI*, the DNA was electrophoresed on a 0.9% agarose gel before being Southern blotted and hybridised with a ^{32}P labelled F-MuLV probe, (a molecularly cloned complete F-MuLV genome in pBRJ22, pF-MuLV, Oliff *et al.*, 1980) and pBBFMUE. Figure 25, shows the restriction pattern obtained from such an analysis. From this it was thus possible to construct a physical restriction enzyme map, with the help of a previously cloned F-MuLV (Oliff *et al*,. 1980) and also from work from this laboratory (Frisby and Ostertag, unpublished). This restriction enzyme map is depicted in Figure 26. The orientation of the respective fragments, and the subsequent genomic location was determined by removing the pF-MuLV


M
a
b
11·9 -
8·1 -
6·7 -
6·1 -
4·5 -
A

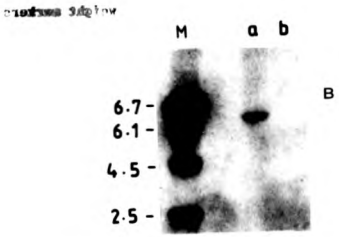

M
a
b
6·7 -
6·1 -
4·5 -
2·5 -
B

Figure 24. Isolation of linear F-MuLV and F-SFFVpM extrachromosomal DNA

DNA samples were electrophoresed in a 0.8% agarose gel in the presence of 0.5 µg/ml EtBr for 12 h before being Southern blotted to a nitrocellulose filter and hybridised with the respective probe.

A. Lane a: appr. 7 kb DNA.
Lane b: appr. 9 kb DNA.
Hybridised with the pBBFMUE probe.

B. Lane a: appr. 7 kb DNA.
Lane b: appr. 9 kb DNA.
Hybridised with the pBRIE probe.

The numbers to the left represent molecular weight markers in kb.

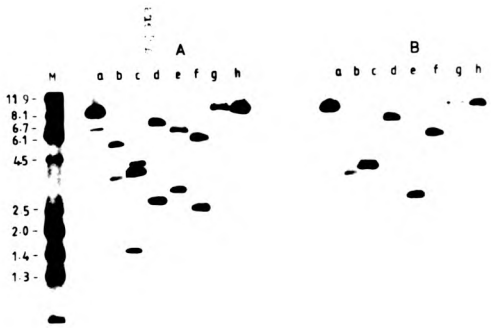

A
B
M
a b c d e f g h
a b c d e f g h
11.9 -
8.1 -
6.7 -
6.1 -
4.5 -
2.5 -
2.0 -
1.4 -
1.3 -

Figure 25. Restriction enzyme analysis of the isolated F-MuLV linear DNA

Aliquots of the isolated F-MuLV linear DNA were digested with restriction enzymes, before being analysed by agarose gel electrophoresis and Southern blotting.

Lane a: uncut.

Lane b: SpHI.

Lane c: HindIII.

Lane d: EcoRI.

Lane e: XbaI.

Lane f: SstI.

Lane g: SalI.

Lane h: XhoI.

A, was hybridised with the F-MuLV probe (pF-MuLV 57).

B, was hybridised with the pBBFMUE probe.

The numbers to the left represent molecular weight markers in kb.

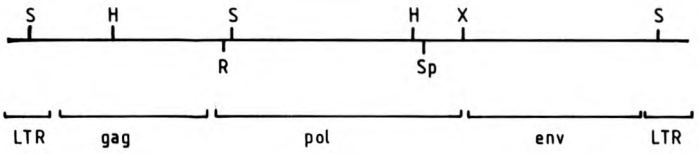

S
H
S
H
X
S
R
Sp
LTR
gag
pol
env
LTR
1kb

Figure 26. Restriction map of isolated F-MuLV linear DNA

The restriction enzyme lettering code is as follows.
S, SstI; H, HindIII; R, EcoRI; Sp, SpHI; X, XbaI.
This restriction enzyme map was constructed from the data presented in Figure 25 and also from unpublished data from this laboratory (Frisby and Ostertag unpublished data). The SstI sites were allocated to the LTRs from analysis of an integrated F-MuLV genome in NRK cells which gave fragments of approximately 6.0 kb and 2.8 kb, thus indicating SstI sites in both of the LTRs as well as one SstI site within the genome. This is also in agreement with data for most ecotropic LTR sequences where a SstI site is highly conserved.

57 probe (Figure 25A) and rehybridising with the env specific pBBFMUE probe (Figure 25B). From this one can see that the smaller 3.75 kb SpHI fragment corresponds to the 3' (env) part of the F-MuLV genome along with the 3.9 kb HindIII, 5.9 kb SstI and 6.6 kb EcoRI fragments. In this experiment the enzymes SalI and XhoI were shown to be non-cutters.

ii) F-SFFVp linear DNA.

A similar approach as shown above was used to map restriction enzyme sites to the isolated F-SFFVp linear DNA using the enzymes EcoRI, HindIII, XhoI, ClaI, SstI, SalI, SpHI, and XbaI. The results of this analysis are shown in Figure 27 (A and B). Results from this analysis show that the enzymes SalI, XhoI and XbaI are non-cutters (as in the F-MuLV genome except for XbaI which cuts at the start of the env gp70 gene). Again two different probes, notably pBRIE, and a HindIII total SFFVp probe, were used to locate various fragments to env locations (B), or to the rest of the genome.

Hybridising with the HindIII probe (Figure 27 A), demonstrated that the uncleaved SFFVp linear DNA is approximately 7.0 kb, as already seen earlier. Digestion with the restriction enzyme EcoRI, gives rise to three fragments of 3.1 kb, 1.8 kb and 1.75 kb, (a double band) of which the 1.8 kb band is the only one hybridising to the env specific probe pBRIE. This 1.8 kb fragment corresponds to an internal env fragment, for, previous restriction enzyme analysis of integrated SFFVp in Friend cells (notably F4-6), this 1.8 kb EcoRI fragment was a characteristic for the Mirand strain of SFFVp, (Frisby and Ostertag, unpublished results). The results of this analysis are depicted as a restriction map in Figure 28.

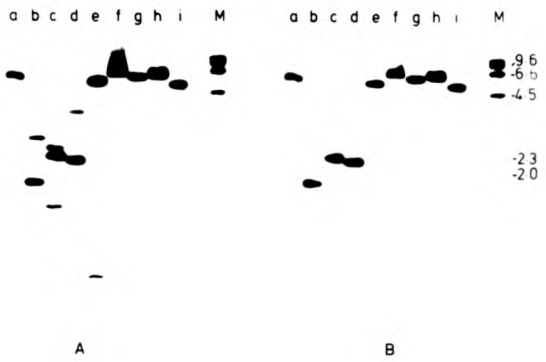

a b c d e f g h i M
a b c d e f g h i M
9.6
6.5
4.5
2.3
2.0
A
B

Figure 27. Restriction enzyme analysis of the F-SFFVpM linear DNA

Aliquots of the isolated linear DNA were digested to completion with various restriction enzymes before being subjected to agarose gel electrophoresis and Southern blotting.

Lane a: uncut.

Lane b: EcoRI.

Lane c: HindIII.

Lane d: SpHI.

Lane e: ClaI.

Lane f: XbaI.

Lane g: XboI.

Lane h: SalI.

Lane i: SstI.

Lane j: molecular weight numbers in kb.

A. Hybridised with the HindIII SFFVp probe.

B. Hybridised with the pBRIE probe.

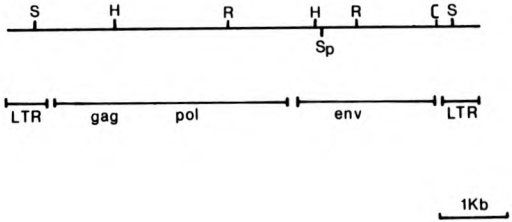

S
H
R
H
R
C
S
Sp
LTR
gag
pol
env
LTR
1Kb

Figure 28. Restriction enzyme map of the linear F-SFFVpM DNA

The map is drawn to scale.

The restriction enzyme lettering code is as follows:

S, SstI; H, HindIII; R, EcoRI; Sp, SpHI; C, ClaI.

XbaI, XhoI and SalI are non-cutters.

This restriction enzyme map was constructed from the data presented in Figure 27 and also from unpublished data from this laboratory (Frisby and Ostertag unpublished data). The SstI sites seen within the LTRs were deduced in a similar way as in Figure 26 after restriction enzyme analysis of an integrated SFFVpM genome in NRK cells (Frisby and Ostertag, unpublished data).

Discussion

Data have been presented that extrachromosomal DNA of the FV complex are present in FLC and that these DNAs (notably a SFFVp component) increase upon treatment of these cells with DMSO. This increase in both the linear and covalently closed circular extrachromosomal DNA molecules, however is only observed in those cells that are able to differentiate into a haemoglobin producing cell (as determined by benzidine staining). In those cells that are refractory to the effects of DMSO (i.e. non-inducible) no change was seen in the amounts of SFFVp extrachromosomal DNA. Although extrachromosomal DNAs of F-MuLV were also identified, they appeared not to be amplified by DMSO treatment in any of the cells analysed.

The presence of SFFVp specific extrachromosomal DNA (notably the linear DNA only) in FLC was first shown by Ikawa et al., (1980), although they did not compare amounts found in undifferentiated and differentiated cells. They also did not observe the presence of extrachromosomal F-MuLV DNA, although these FLCs release high virus titres of SFFVp as well as F-MuLV.

More recent experiments by Kern and Axelrod (1983) looked in more depth at this observation. In their studies they used derivatives of the 745A line which had been derived by sib-selection in vitro for resistance, or sensitivity to the inducing effects of DMSO (Axelrod et al., 1979). Upon analysing these cells, they found that there was a greater than eight fold variation in the amount of SFFV extrachromosomal DNA present, with the largest amounts being found in cells that were resistant to the induction of differentiation by DMSO. These results conflict with those presented in this study where the

reciprocal is the case. However the cells used by Kern and Axelrod all stem from the same parent, 745A. In the study presented here, the cell lines analysed in detail, notably F4-6, F4N+2, F4+ and B8/3 are all individually isolated clones that have not been selected for their resistance to DMSO, (although B8/3 is a BUdR selected clone of F4 from which F4-6 is derived). These cells thus represent mutants that are blocked in their differentiation capacity (either precommitted or committed) per se, and not cells that are selected in vitro by their resistance to the inducing effects of DMSO. This could possibly contribute to the explanation of these conflicting results.

Results, only mentioned, and not further discussed in the study of Kern and Axelrod, also state that they had also isolated cell lines, that did not show the correlation which they presented as their main argument. Further queries can also be posed about the 745A cell line used, which according to the literature is transformed by the FV-A strain of Friend virus and also releases this virus, (Friend et al., 1971; Golde et al., 1979), although the authors claimed that the extrachromosomal DNA seen was of a polycythaemia strain.

A further observation, i.e. there is an immediate decrease in the amount of extrachromosomal SFFV DNA, related to the transient delay in the G1 phase of the cell cycle (that is observed in FLCs after the addition of DMSO to the growth medium, Friedman and Schildkraut, 1978; Terada et al., 1977), is also seen in the cell lines F4-6 and F4N+2. That no (or very little) extrachromosomal DNA is seen in F4-6 and F4N+2 after 96 h p.d.i. could be explained by the fact that the growth arrest in these terminally differentiated cells (Gusella et al., 1976) leads to the cessation in synthesis of the extrachromosomal DNA.

The cytoplasmic location of these extrachromosomal DNAs, in the cell

line F4-6, corresponds to that found by Kern and Axelrod. What is unknown is the stimulus for synthesis of this extrachromosomal DNA. It is possible that the elevated extrachromosomal SFFV DNA reflects increased viral transcription and secondary reverse transcription similar to a system observed in dexamethasone-treated rat hepatoma cells infected with MMTV (Ringold et al., 1978). The possibility was considered by Kern and Axelrod that the effects of prolonged DMSO treatment on the cell membrane result in an increased rate of superinfection. However, if this were the case one would expect to see new integration sites in these superinfected cells. In both the studies presented here (data not shown) and those conducted by Kern and Axelrod, no new integration sites could be detected, suggesting that this was indeed not the case. Such events, i.e. reintegration, have been seen in HTLV-1 infected human HL60 cells, after passage in vitro. (Hiramatsu et al., 1988).

To further explain their results, Kern and Axelrod suggested two possibilities.

i) Continual selection for pre-existing subpopulations of cells that contain increased amounts of extrachromosomal SFFV DNA.

ii) Increased synthesis or maintenance of this DNA associated with the prolonged exposure of DMSO required for the transition from DMSO-sensitivity to DMSO-resistance.

In the study of Kern and Axelrod both of these possibilities could hold true for the cells used were selected for either resistance or sensitivity to the stimulating effects of DMSO. However in the experiments presented here extrachromosomal DNAs were seen in cells that could be induced by DMSO, thus suggesting that the hypothesis presented above is not generally applicable.

One intriguing observation is that they detected no helper related sequences, as found here, but they did not mention whether the 745A derivative used actually produced virus or not. Also is the study described here no extrachromosomal DNA whatsoever in the cell line B8/3, which has a defect helper virus function, suggesting that possible helper virus functions could assist in the synthesis of this extrachromosomal DNA, although at the moment no mechanism can be suggested.

The finding of extrachromosomal DNAs (FV specific) only within FLC and not in non-producer or producer fibroblast cell lines of either murine or non-murine origin suggests that they may be restricted to FLC or cells of erythroid nature. Examination of chemically induced rat erythroleukaemia cell lines (Kluge et al., 1976) infected with FV-P could further clarify this point.

These experiments were designed as a pre-requisite to obtain information as to which cell line would be most suitable as a source of the extrachromosomal DNA (in particular circular) for molecular cloning. The cell line F4-6 was selected as it reproducibly synthesized detectable amounts of both F-MuLV and F-SFFVp extrachromosomal DNA.

It was also interesting to see whether it was possible to molecularly clone an "induced-DNA" of the F4-6 SFFVp. Large scale preparation of extrachromosomal DNA from (72 h p.d.i.) F4-6 cells followed by CsCl gradient fractionation and finally preparative agarose gel electrophoresis led to the isolation of both the F-MuLV and F-SFFVp linear DNAs. The restriction enzyme digestion and Southern blot analysis using specifically cloned probes enabled the construction of primary restriction enzyme maps.

Chapter 2

Molecular cloning of the Friend virus complex

Introduction

Although many SFFV strains have been cloned, including F-SFFVa, (Kaminchik et al., 1982) various strains of F-SFFVp (Linemeyer et al., 1980; Yamamoto et al., 1981; Amanuma et al., 1983), as well as the R-SFFV (Bestwick et al., 1984; Hess et al., 1984), it was still very interesting to molecularly clone the Mirand strain of F-SFFVp (Mirand, 1968), for two reasons. The approach which was to be adopted, as outlined in the previous chapter, meant that both the F-MuLV and F-SFFVp, would be molecularly cloned from the same cell in which it has been previously shown that the helper virus and SFFVp had indeed co-evolved very closely (Mol et al., 1981). All other cloning experiments have cloned either the helper virus or the SFFV, from producer or productively infected cells, in which the two viruses had thus been separated, and in some cases for long periods (up to several years), thus allowing for the two viral components to molecularly diverge. The genetic heterogeneity of F-SFFVp is most likely a consequence of two processes. New recombinations of the SFFVp genome with replication competent mink cell focus forming virus (MCFV) or MuLV and accumulation of mutations in those parts of the genome which are not required for biological activity. Recombination between SFFV and helper virus sequences can be inferred from the data of Mol et al., (1981) and Obata et al., (1984).

Whether the genetic heterogeneity of F-SFFVp is also of biological importance is at present uncertain. An interesting indication of biological divergence of F-SFFVp variants is still unexplained. Most F-SFFVp variants induce a large increase in CFU-E density in the spleen

and only a very small increase, if at all in the bone marrow (Liao and Axelrad, 1975; Peschle et al., 1980). This is different for a F-SFFVp complex (notably the Mirand strain) released by some Friend cells and cloned in fibroblasts (Fagg et al., 1980; Ostertag and Pragnell, 1981; Fagg and Ostertag, 1982).

Results

a) Molecular cloning strategy

The results obtained in the previous chapter enabled the construction of primary restriction enzyme maps, which could thus be used to select for an enzyme for the molecular cloning of the F-MuLV and F-SFFVp extrachromosomal DNAs, and also to identify such positive clones on hand from specific restriction enzyme fragments. Thus as it was proposed to molecularly clone the circular DNAs of these two viruses using a restriction enzyme which cuts only once within the genome of the two viruses. The restriction enzyme SpHI was chosen for this purpose, for it cuts only once (as far as could be determined from the restriction maps obtained and also from published sequence data, where it appears that a single SpHI site is conserved in both F-MuLV and SFFV, Amanuma et al., 1983; Clark and Mak, 1983; Wolff et al., 1983; Adachi et al., 1984; Koch et al., 1984; Koch et al., 1984; Wolff et al., 1985). According to this analysis it was decided to molecularly clone the SpHI digested viral DNAs into the plasmid cloning vectors pUC19 and pBR322, before transforming the recombinant plasmids into competent bacteria, prepared by the Hanahan procedure (Hanahan, 1983, 1985) a method which has been successfully applied to the molecular cloning of various other SFFVs (Linemeyer et al., 1980; Kaminchik et

al., 1982; Amanuma et al., 1983; Bestwick et al., 1984).

One requisite for this approach was to have highly competent bacteria so that a representative number of recombinant clones could be obtained. Thus as a starting point batches of competent cells (DH1 and DH5α) were prepared, frozen and then tested in their transformation efficiency. These bacterial strains were chosen for they are known to give highly competent cells, after preparation according to Hanahan. The results of this analysis are shown in Table 4.

From these experiments it can be seen that the two batches of cells from experiment 3 (DH1, 1 x 10^7) and experiment 10 (DH5α, 4 x 10^7) gave the highest transformation frequencies, at an order of efficiency required for such a cloning experiment. DH5α had an additional advantage over DH1 in that one is able to determine the percentage of recombinant plasmids in the presence of X-gal and IPTG when used in conjunction with pUC19. A second plasmid cloning vector pBR322 was used in parallel.

b) Construction of the library

CsCl fractionated circular, F-MuLV and F-SFFVp DNA (i.e. those found in fraction 4 in Figure 23, 100 ng/ml) was digested to completion with the restriction enzymes SpHI, XhoI and SalI, and after phenol/chloroform extraction was ethanol precipitated before being resuspended at a concentration of 1 µg/ml in TE solution. The XhoI and SalI digestion step was performed so as to digest non-specific DNA containing these restriction enzyme sites giving non-compatible ends, which would then not be able to form viable recombinant plasmids.

The ligation of SpHI digested and calf-intestinal phosphatase treated pUC19 and pBR322 (in a total volume of 10 µl) was performed at various

Table 4. Preparation of competent cells for molecular cloning

Experiment	Cells used	Transformation efficiency colonies/µg pBR322
1	DH1	1×10^6
2	DH1	5×10^6
3	DH1	1×10^7
4	DH1	1×10^8
5	DH1	5×10^7
6	DH5α	1×10^7
7	DH5α	5×10^8
8	DH5α	1×10^6
9	DH5α	3×10^7
10	DH5α	4×10^7

Cells were prepared as in Materials and Methods (Hanahan, 1985) and then frozen and stored at -70°C.

All preparations were tested at the same time on the same batch of agar plates, so as to avoid variables.

Transformations were as described in Materials and Methods.

vector to insert ratios, as described in Materials and Methods, before being transformed into either competent DH1 or DH5α. The results are shown in Table 5.

c) Screening of the library

i) Identification of positive clones

The colonies obtained (which had been plated out on Nunc 20x20 cm LB ampicillin plates, either with or without X-gal and IPTG depending on the cloning vector and bacteria used) were then screened for positive recombinants by the method of Grunstein and Hogness (1975).

Replica nitrocellulose filters were hybridised with the F-SFFVp HindIII ^{32}P-probe which hybridises to both F-MuLV and F-SFFVp related sequences. An autoradiogram from one pair of replica filters is shown in Figure 29. A total of 10 positive clones were detected by this procedure, which were designated pcl-1 to pcl-10. Rehybridisation of the washed filters with the F-MuLV specific probe, (the BBFMUE 700 bp insert) revealed that two clones, pcl-5 and pcl-10, hybridised with this fragment. However, after removal of the pBBFMUE probe and rehybridisation for a third time with a ^{32}P labelled BRIE fragment, no positive colonies were detected.

Thus from the primary screening of the plasmid library obtained, it appeared that there were two F-MuLV related clones, and eight clones which hybridised only with the F-SFFVp genomic probe, and not with either of the two *env* specific probes. Of the ten positive clones obtained, only one (pcl-8) was obtained with the pBR322, DH1 combination. All others were in pUC19 and DH5α.

Table 5. Construction of plasmid libraries to clone the circular F-MuLV and F-SFFVpM

Ligation conditions		Transformation results	
Vector (pUC19) (µg)	Insert (µg)	Total number of colonies	white DH5α (%)
0.1	0.5	382	90
0.1	1	867	88
0.1	2	1727	95
0.1	5	1238	92
Vector (pBR322) (µg)	Insert (µg)	Total number of colonies	Tet^R DH1 (%)
0.1	0.5	90	5
0.1	1	93	3
0.1	2	273	6
0.1	5	159	5

Controls

Vector (100 ng)	DH5α	DH1
pUC19.SpHI.CIP	50	-
pUC19.uncut	$3x10^7$	-
pBR322.SpHI.CIP	-	20
pBR322.uncut	-	$2.5x10^7$

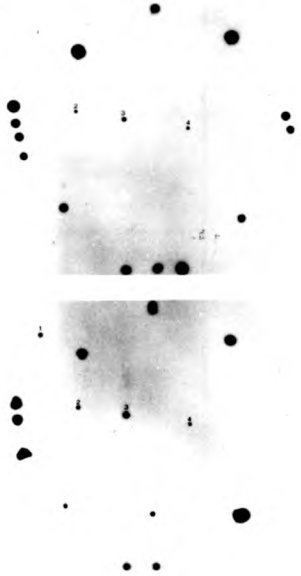


Figure 29. Screening of bacterial colonies for recombinant plasmids by in situ hybridisation (Grunstein and Hogness blots)

Depicted are the autoradiographs of replicate filters, lifted from a 20 x 20 cm LB ampicillin plate onto which pUC19 recombinants in DH5α had been plated. The filters were hybridised as in Materials and Methods with a ^{32}P labelled SFFVp, HindIII probe.

The numbered signals represent recombinant clones, which were visible on both films. Other signals represent orientation markers.

Exposure was at -70°C for 24 h.

d) Molecular analysis of F-MuLV clones

Small scale plasmid DNA preparations, from the clones pcl-5 and pcl-10, revealed that both contained approximately 9 kb inserts after digestion with the restriction enzyme SpHI followed by agarose gel electrophoresis. These clones contained a 9 kb fragment, which hybridised to the F-MuLV specific BBFMUE probe as seen above. Further digestion of these plasmids with the restriction enzymes HindIII and BamHI revealed that both of these clones contained an identical cloned insert in the same orientation. Further analysis was carried out on the pcl-5 clone.

i) Restriction enzyme mapping of pcl-5

To further identify the nature of the clone pcl-5, the bacteria were expanded and CsCl gradient purified plasmid DNA was prepared. The plasmid DNA was then analysed by restriction enzyme mapping, agarose gel electrophoresis and Southern blotting. The results of this primary analysis are depicted in Figure 30. This analysis demonstrated that the insert cloned in pcl-5 was a permutated circular copy of the F-MuLV linear DNA described in Chapter 1 (Figure 26). This conclusion can be drawn, from the size of the restriction enzyme fragments, which hybridised to the F-MuLV env specific probe (BFFMUE) i.e. cloning of the circular molecule, by SpHI digestion into the corresponding site into pUC19, and then digestion of this permutated copy with SstI would give rise to either 2.85 or 5.9 kb env specific fragment depending on the orientation.

This primary analysis thus positively identified the nature of the DNA molecule cloned in pcl-5 (identical results were obtained for pcl-10, thus substantiating the conclusion presented earlier) and also

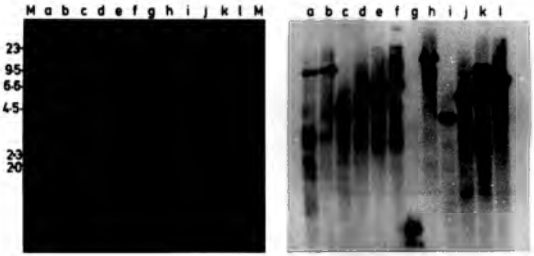

M a b c d e f g h i j k l M
23
95
66
45
23
20
a b c d e f g h i j k l

Figure 30. Restriction enzyme analysis of the F-MuLV recombinant clone pcl-5

The plasmid pcl-5 was analysed by a combination of restriction enzyme digestion and Southern blotting. Plasmid DNA (1 µg) was digested to completion before being subjected to agarose gel electrophoresis (1% gel in 0.5x TBE buffer at 150 V for 3 h in the presence of ethidium bromide). After photographing the ethidium bromide stained gel, the DNA fragments were transferred to a nitrocellulose filter by the method of Southern. The filter was then hybridised with a ^{32}P labelled F-MuLV *env* specific probe BBMFUE.

Lane M; lambda DNA digested with *HindIII* in kb.

Lane a; *SpHI*.

Lane b; *EcoRI*.

Lane c; *HindIII*.

Lane d; *PstI*.

Lane e; *SstI*.

Lane f; *KpnI*.

Lane g; *BamHI*.

Lane h; *SalI*.

Lane i; *SmaI*.

Lane j; *PvuII*.

Lane k; *EcoRI*.

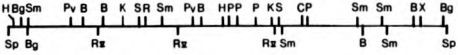
Lane l; *BglII*.

gave further information for the further analysis of this clone. From the analysis here (and previously, Chapter 1) it can be seen that SalI does not cut within the pcl-5 insert, but cuts once within the polylinker cloning region of pUC19. Similarly ClaI cuts only once within the pcl-5 insert and not at all within the pUC19 plasmid vector. This information was important for the restriction enzyme mapping which was performed according to a method developed by Smith and Birnstiel (1976). In this method the DNA molecule to be analysed, is labelled at one end with ^{32}P and then partially digested with the desired restriction enzymes. DNA fragments are obtained which form an overlapping series of molecules, all with a common labelled terminus. A restriction map can then be constructed from the analysis of the size distribution of these molecules.

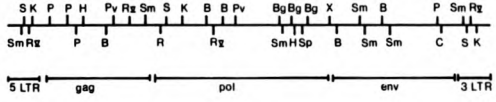
Thus pcl-5 was digested to completion with SalI and the free ends were then labelled with ^{32}P γ-ATP using polynucleotide kinase (as described in Materials and Methods). The ^{32}P labelled linear DNA was then digested to completion with the enzyme ClaI giving rise to two DNA fragments of 4.55 kb and 5.15 kb, which were separated and isolated by preparative agarose gel electrophoresis and electroelution. These labelled fragments were then used for the detailed restriction enzyme mapping procedure described above. This analysis enabled the construction of a detailed restriction enzyme map (Figure 31).

e) Molecular analysis of F-SFFVp

From the molecular screening procedure it was seen that, excluding pcl-5 and pcl-10 (which were demonstrated to be molecularly related to F-MuLV), there were eight further clones which, although they did not hybridise to env specific probes, did hybridise to the F-SFFVp HindIII


H Bg Sm Pv B B K SR Sm Pv B HPP P KS CP Sm Sm BX Bg
Sp Bg Rɪ Rɪ Rɪ Sm B Sm Sp

A


S K P P H Pv Rɪ Sm S K B B Pv Bg Bg Bg X Sm B P Sm Rɪ
Sm Rɪ P B R Rɪ Sm H Sp B Sm Sm C S K
5 LTR gag pol env 3 LTR

B

1Kb

Figure 31. Detailed restriction enzyme map of pcl-5

From the analysis of the clone pcl-5 (and also for pcl-10 which is identical) as detailed in the text, a detailed restriction enzyme map was constructed. The map of the permutated copy, cloned at the SpHI site is shown in A.
A linear map delineating the genome is shown in B.

Restriction enzyme lettering code:
B, BamHI; Bg, BglII; C, ClaI; H, HindIII; K, KpnI; P, PstI; Pv, PvuII; R, EcoRI; Rv, EcoRV; S, SstI; Sm, SmaI; Sp, SpHI; X, XbaI.

probe. Restriction enzyme analysis of small scale plasmid preparations from these clones (pcl-1 to pcl-4 and pcl-6 to pcl-9) as well as detailed restriction enzyme analysis of CsCl gradient purified plasmid DNA, showed that all of these clones represented rearranged (deleted) versions of DNA molecules that were similar to the F-SFFVpM linear DNA described in Chapter 1 (Figure 28).

Restriction enzyme maps of these clones (Figure 32) demonstrated that all of these clones appeared to be deleted versions of F-SFFVpM, and that all deletions were located within or encompassed the presumptive env (gp55) region. Indeed in some cases even part of the polylinker, including the SpHI site had been deleted (as shown by linearisation of the plasmids, after digestion with SpHI).

Why or how these deletions occured is somewhat unclear, although cloning within pUC19, which is a prokaryotic expression vector, when in the correct reading frame can give rise to a β-galactosidase fusion protein, which may prove toxic for the bacteria. Indeed similar problems have been encountered, when subcloning SFFV env specific sequences into the related pUC9 vector (N. Hess, pers. commun.) and also when cloning a novel ras containing F-MuLV recombinant virus (J. Friel, pers. commun.). It may also be that sequences within this region are poisonous per se or prone to recombination in the bacteria used in this study.

f) Molecular cloning of F-SFFVpM in a bacteriophage lambda

Due to the unforseen difficulties encountered with the plasmid cloning strategy of F-SFFVpM (although successful for F-MuLV, pcl-5, and indeed other SFFVs) it was decided to develop a new strategy for the cloning of the circular extrachromosomal DNA which had been

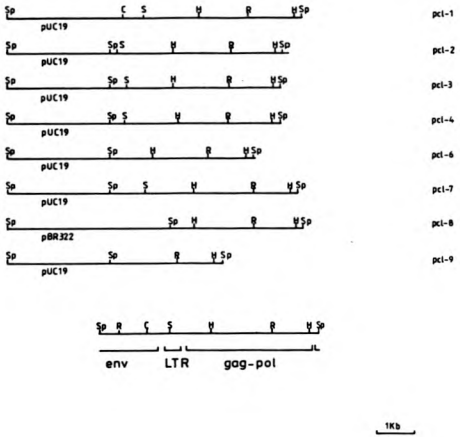

Sp C S H B H Sp
pUC19
pci-1
Sp Sp S H B H Sp
pUC19
pci-2
Sp Sp S H B H Sp
pUC19
pci-3
Sp Sp S H B H Sp
pUC19
pci-4
Sp Sp H B H Sp
pUC19
pci-6
Sp Sp S H B H Sp
pUC19
pci-7
Sp Sp H B H Sp
pBR322
pci-8
Sp Sp B H Sp
pUC19
pci-9
Sp B C S H B H Sp
env
LTR
gag-pol
1Kb

Figure 32. Restriction enzyme maps of recombinant F-SFFVpM plasmid clones

All plasmid DNA from clones, pcl-1 - pcl-4 and pcl-6 - pcl-9 were purified by two rounds of CsCl equilibrium centrifugation, before being subjected to restriction enzyme digestion and agarose gel electrophoresis. The clones were analysed by digestion with the restriction enzymes;

C, ClaI; H, HindIII; R, EcoRI; S, SstI and Sp, SpHI.

All maps are drawn to scale.

previously isolated. To this aim a new strategy was planned, which involved molecular cloning in a bacteriophage lambda cloning vector: Molecular cloning of the virus in two fragments. From the restriction map of the F-SFFVpM linear DNA, it was shown that there are two HindIII restriction enzyme sites within the F-SFFVpM genome. Thus digestion of a circular F-SFFVpM DNA molecule would give rise to two fragments of approximately 2.5 kb and 4.0 kb.

The isolated circular F-SFFVpM extrachromosomal DNA (which also contained F-MuLV circular forms) was digested to completion with HindIII and ligated to HindIII digested and dephosphorylated Charon 21A DNA (arms), the details of which are in the Methods. The ligation reactions were then packaged in vitro resulting in an infectious recombinant phage library. This library was then screened (at a density of 10^5 plaques/ 20x20 cm dish) by the method of Benton and Davies (1977). Replica filters were hybridised with the HindIII F-SFFVp probe as described in Materials and Methods. From the primary screening of 4 x 10^4 plaques, twenty four gave positive hybridising signals on replica filters. These positive plaques were picked and purified by a second round of low density plaque screening. Single, positive plaques were picked, and small scale DNA preparations of these clones, were analysed by agarose gel electrophoresis and Southern blotting after digestion of the recombinant lambda DNA with HindIII. Hybridisation with the HindIII F-SFFVp probe revealed that twenty two of these lambda clones did contain SFFV related sequences either 4 - 4.5 kb in size (9 clones designated L 4#1 - L 4#9) or 2.5 kb fragments (13 clones L 2#1 - L 2#13). To further analyse these clones, the recombinant lambda DNA was analysed as above except that the Southern blots were hybridised with SFFV env and gag specific probes. These probes were

chosen for according to the linear map constructed of F-SFFVpM from the data in Chapter 1 then the approximately 4.5 kb F-SFFVpM HindIII fragment should hybridise to the BRIE probe, whereas both the approximately 4.5 kb and 2.5 kb fragments should hybridise to a gag specific probe. This analysis showed that clones L 2#4, L 4#5, L 4#6 and L 4#7 hybridised to the BRIE probe whereas L 2#1, L 2#3, L 2#4, L 2#8, L 2#9, L 2#12, L 4#9, L 4#6, L 4#5 and L 4#4 hybridised to the gag specific probe.

The remaining DNA from the small scale lambda DNA preparations was digested with HindIII and after separation of the DNA fragments by agarose gel electrophoresis, the recombinant DNA inserts were isolated by electroelution and subcloned into HindIII digested pUC9 cloning vector. After transformation into competent NM522 bacteria, small scale plasmid preparations were performed on the recombinant clones.

These bacteria were chosen for they overproduce the lac repressor, which binds to the promoter region in the pUC plasmids thus repressing expression of genes cloned in the polylinker region. However, this repression is not absolute. Clones containing the correct inserts were then analysed by a combination of restriction enzyme mapping and Southern blotting after purifying the plasmids by two rounds of CsCl gradient centrifugation. The restriction maps assembled from this analysis are depicted in Figure 33.

The clones p2#1, p2#3, and p2#8 all have the same structure as determined by digestion with the enzymes EcoRI and BamHI and that this preliminary restriction enzyme map agrees with that described by the analysis in Chapter 1. All three of these clones hybridised with the gag fragment and not with the BRIE specific probe, thus confirming the internal origin of this HindIII fragment. However, three further

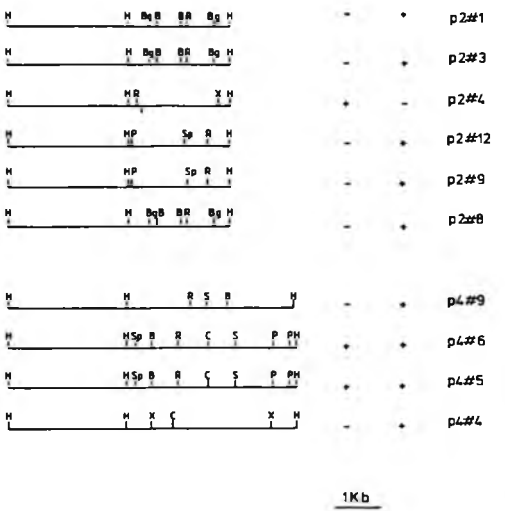

hybridisation with clone
env
gag
H
H BgB BR Bg H
p2#1
p2#3
HR X H
p2#4
HP Sp R H
p2#12
p2#9
p2#8
R S B
p4#9
HSp B R C S P PH
p4#6
p4#5
H X C X H
p4#4
1Kb

Figure 33. Restriction enzyme maps of subcloned F-SFFVpM HindIII fragments

Depicted in this diagram are scale maps of the HindIII subgenomic fragments cloned in pUC19. Also shown are the hybridisation data with the env specific (pBRIE) and gag specific probes.

Restriction enzyme lettering code:

B, BamHI; Bg, BglII; C, ClaI; H, HindIII; P, PstI; R, EcoRI; S, SstI; Sp, SpHI; X, XbaI.

clones, notably p2#4, p2#9 and p2#12, which hybridised to either the gag (p2#9 and p2#12) or BRIE probe (p2#4) had somewhat different restriction enzyme maps as compared to the others. The nature and origin of these fragments is unclear for they could not be assigned to either the F-SFFVpM or the F-MuLV genome.

Analysis of the clones p4#9, p4#6, p4#5 and p4#4, also showed that there was a heterogeneity in the fragments that had been cloned. Of the clones that hybridised with the gag specific probes, two, p4#6 and p4#5 corresponded to the second HindIII fragment produced after digestion of the F-SFFVpM circular form as confirmed by their hybridisation to the BRIE probe. Two further clones p4#9 and p4#4 had different restriction enzyme patterns. On further analysis p4#9 could be identified as the internal F-MuLV HindIII fragment when compared with the restriction map of pcl-5. Clone p4#4 which hybridised to the gag probe, had a restriction enzyme pattern which was non-identical to either that of pcl-5 (F-MuLV) or F-SFFVpM.

g) Molecular analysis of the molecularly cloned Mirand strain F-SFFVpM.

i) Construction of a restriction enzyme map for F-SFFVpM

Using the different cloning procedure described above, the two HindIII fragments specific for F-SFFVpM had been successfully cloned. These two fragments (using the clones p2#1 and p4#6) were then subjected to a detailed restriction enzyme analysis using the methodology of Smith and Birnstiel (1976). This analysis thus enabled the construction of a detailed restriction enzyme map representing the F-SFFVpM proviral DNA linear molecule.

ii) Comparative analysis of F-SFFVpM with other molecularly cloned SFFVs

Having a detailed restriction enzyme map of F-SFFVpM thus enabled the comparative analysis of the DNA proviral genome with other molecularly cloned SFFVs as depicted in Figure 34. In this figure F-SFFVpM is compared with three strains of F-SFFVp as well as F-SFFVa and R-SFFV. The first point to note from this analysis is that the Mirand strain of F-SFFVp although having similar biological properties as the other SFFVps and although similar at the genome level (as determined by hybridisation with specific probes) is also unique (as seen by the restriction enzyme map) thus showing divergence of this strain as compared to the others. F-SFFVpM is approximately the same size as the Axelrod (Ax) and the Lilly and Steeves (LS) SFFVp's with a linear DNA proviral genome of 6.5 kb. Although various restriction enzyme sites are shared between the different SFFV strains, including F-SFFVpM, it is only when one examines the env gene that a similarity becomes apparent. In nearly all of the SFFVs depicted above there is an area of approximately 1 kb which appears to be conserved to a high degree in most of the gp55 env genes (determined by sequence analysis of the other SFFVs depicted here), centered around the SmaI, EcoRI and PvuII restriction enzyme sites. This is based on sequence analysis of previously cloned SFFVs. This part of the gp55 gene corresponds to the region which is of non-ecotropic origin i.e. from MCF or endogenous mouse sequences and are indeed sequences which are used to diagnose SFFV specificity both at the nucleotide and protein levels. Comparison of the rest of the genome, using the restriction enzyme sites shows that at this level there is very little homology which is not surprising when one considers that SFFVs are recombination products

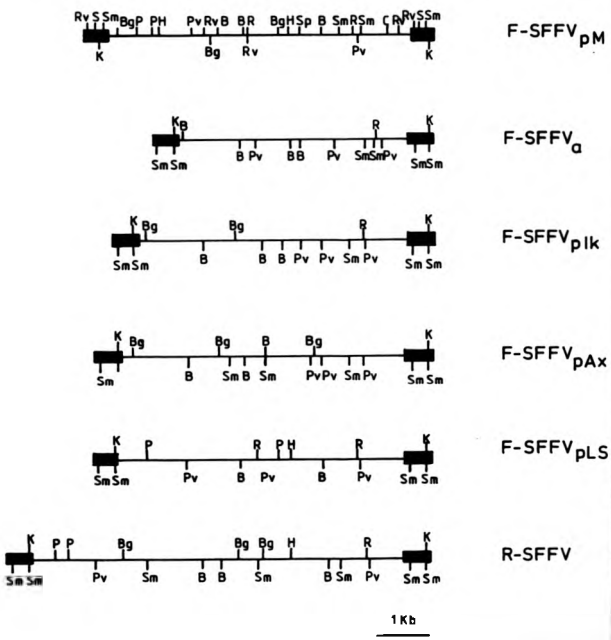

Rv S Sm Bg P PH Pv Rv B BR Bg H Sp B Sm R Sm C R Rv S Sm
K Bg Rv Pv K
F-SFFV pM
K B R K
Sm Sm B Pv B B Pv Sm Sm Pv Sm Sm
F-SFFV a
K Bg Bg R K
Sm Sm B B B Pv Pv Sm Pv Sm Sm
F-SFFV plk
K Bg Bg B Bg K
Sm B Sm B Sm Pv Pv Sm Pv Sm Sm
F-SFFV pAx
K P R P H R K
Sm Sm Pv B Pv B Pv Sm Sm
F-SFFV pLS
K P P Bg Bg Bg H R K
Sm Sm Pv Sm B B Sm B Sm Pv Sm Sm
R-SFFV
1 Kb

Figure 34. Comparison of the restriction enzyme map of F-SFFVpM with other molecularly cloned SFFVs

All maps are drawn to scale. The restriction as were adapted from Kaminchik et al., 1982; Wolff et al., 1984: F-SFFVa; Amanuma et al., 1983: F-SFFVpIK; Yamamoto et al., 1981; Clark and Mak, 1983: F-SFFVpAx; Linemeyer et al., 1980; Wolff et al., 1985: F-SFFVpLS; Bestwick et al., 1984; Hess et al., 1984: R-SFFV.

Restriction enzyme lettering code:

B, BamHI: Bg, BglII; C, ClaI: H, HindIII: K, KpnI: P, PstI; Pv, PvuII; R, EcoRI: Rv, EcoRV; S, SstI: Sm, SmaI: Sp, SpHI; X, XbaI.

between ecotropic viral sequences and MCF like env sequences.

h) Biological activity of molecularly cloned F-MuLV and F-SFFVpM

i) F-MuLV: Construction of a linear form of F-MuLV

The results presented previously have shown that two permutated copies (both with one LTR) of F-MuLV (pcl-5 and pcl-10) had been molecularly cloned. Comparison of their (only pcl-5 is presented) genome continuity with F-MuLV 57 showed a certain degree of relatedness. However to further test this it was necessary to determine whether these clones (pcl-5) did indeed have the characteristic biological properties of F-MuLV. To this aim it was then essential to construct a linear form of the pcl-5 clone which could be transfected into recipient cells. The strategy employed in the construction of a linear form of pcl-5 (pcl-5 1L) is depicted in Figure 35. Restriction enzyme analysis of 24 clones with the enzyme SstI revealed that two clones pcl-5 1L#3 and pcl-5 1L#9 gave the correct restriction fragment pattern. These two clones were then expanded and CsCl plasmid DNA was isolated. Further restriction enzyme analysis (Sst, EcoRV and EcoRI.XbaI) substantiated that these clones contained the construct depicted in Figure 35.

ii) Transfection of RAT1 cells

Having constructed a linear F-MuLV it was necessary to construct cell lines which express this construct and thus release replication competent F-MuLV. To this end the following strategy was employed: RAT1 cells were transfected with either pcl-5 1L#3 or pcl-5 1L#9 (as in Materials and Methods) and these transfected cells were then further propagated so that the cells were continually in log phase. After ten

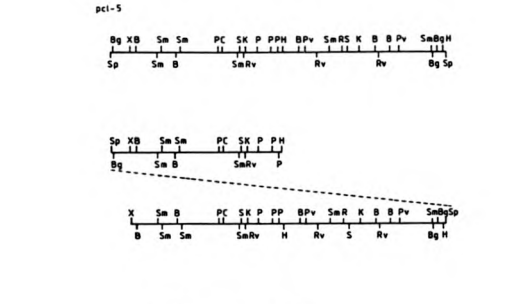

pcl-5
Bg XB Sm Sm PC SK P PPH BPv SmRS K B B Pv SmBgH
Sp Sm B SmRv Rv Rv Bg Sp
Sp XB Sm Sm PC SK P PH
Bg Sm B SmRv P
X Sm B PC SK P PP BPv SmR K B B Pv SmBgSp
B Sm Sm SmRv H Rv S Rv Bg H
ligate with X, H digested pUC19
pcl-5 1L
XB B PC SK P P P B Rv RS K BRvB HSp XB B PC SK P PPH
Rv H Rv
env 5'LTR gag pol env 3'LTR gag
1 Kb

Figure 35. Construction of a linear clone (pcl-5 1L) of the permutated F-MuLV clone pcl-5

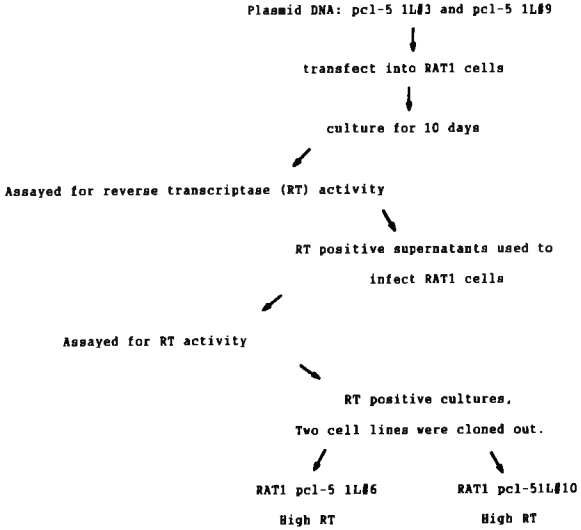
pcl-5 1L (#9) was constructed as follows.
A 4.5 kb HindIII.SpHI fragment, encompassing the 3' end of pol, the complete env and 3' LTR as well as 5' portion of gag, was isolated by preparative gel electrophoresis and electroelution.
A second fragment (XbaI to SpHI, 8.2 kb) was isolated by a similar procedure. The two fragments were ligated in equimolar amounts with XbaI, HindIII digested pUC19. After transformation into competent NM522, ampicillin resistant colonies were screened by restriction enzyme analysis of small scale plasmid DNA preparations.

days of culture the supernatant was tested in the reverse transcriptase assay to see whether replication competent virus was being released. In all cultures, a high reverse transcriptase activity was detected, thus showing that the pcl-5 1L constructs could give rise to replication competent virus. The transfected cells were then further cultured and the supernatants were used to infect RAT1 cells. This step was undertaken to obtain cell lines that only had correctly integrated viral copies, for after transfection of plasmid DNA into cells, there always appears to be a certain amount of recombination, thus leading to aberrant copies. The infected RAT1 cells were then analysed after ten days in culture for the production of replication competent virus (reverse transcriptase assay). All cell cultures analysed released high titres of virus. From one of these cultures cell clones were isolated by plating out the cells at low density and then picking isolated colonies after an incubation period of seven days. Two clones RAT1 pcl-5 1L#6 and RAT1 pcl-5 1L#10, both of which originated from the molecular clone pcl-5 1L#9, and had high RT levels were then used for further analysis. The production of these cell lines is depicted in Figure 36.

iii) Molecular analysis of the producer cell lines RAT1 pcl-5 1L#6

To substantiate that the virus being produced from the cell line RAT1 pcl-5 1L#6 was indeed identical to that, that had been constructed and transfected, the following analysis was performed. Genomic DNA from the cell line RAT1 pcl-5 1L#6 was digested to completion with the enzymes Sst, KpnI, BamHI and EcoRV. The plasmid DNA pcl-5 1L#9 was digested with the same enzymes and both DNAs were then analysed in parallel by Southern blotting. The results of this analysis are shown

Figure 36. The construction of the F-MuLV producing cell lines RAT1 pcl-5 1L#6 and RAT1 pcl-5 1L#10



in Figure 37. Using the pcl-5 SpHI insert fragment as a ^{32}P labelled probe it can be seen that the internal fragments (highlighted by bars) produced after digestion with these enzymes are identical for both the plasmid and the integrated viral DNA. The other bands not marked with arrows represent either, viral plus flanking sequences (integration sites), or non linear sequences of the pcl-5 1L#9 construct (i.e. plasmid sequences etc.).

Thus it appeared that not only does RAT1 pcl-5 1L#6 (similar data not shown was also seen for RAT1 pcl-5 1L#10.) release a replication competent virus, but also that this is of the correct molecular composition.

iv) Biological assay of F-MuLV being released from producer cells

To further substantiate the claim that what had indeed been molecularly cloned and transfected, was indeed F-MuLV, the virus being released from the cell line RAT1 pcl-5 1L#6 was assayed for biological activity both in vitro and in vivo.

To test the biological activity of the virus being released in vitro the XC assay was employed. This method exploits the observation that MuLVs are able to cause the formation of syncitia after infection of RAT XC cells. This method, in combination with the fibroblast cell lines (A31, Balb/c or NIH3T3, NIHSwiss) is also able to define the tropism of the virus being assayed. Thus virus being released from the cell line RAT1 pcl-5 1L#6 was titrated on the A31 and NIH3T3 cells in the XC assay. As a positive control virus being released from the cell line F4-6 was also titrated in parallel. The results are shown in Table 6.

This experiment demonstrated that the virus being released from

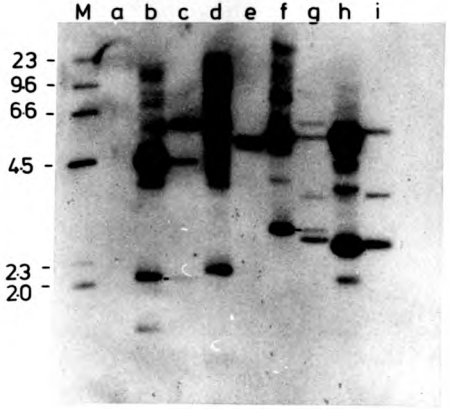

M a b c d e f g h i
23
96
66
45
23
20

Figure 37. Restriction enzyme mapping of the F-MuLV producer cell line RAT1 pcl-5 1L#6

Genomic DNA (10 µg) in parallel with plasmid DNA (pcl-5 1L#9, 80 pg) was digested with various restriction enzymes followed by agarose gel electrophoresis and Southern blotting. The resulting filter was then hybridised, with the SpHI fragment isolated from pcl-5 after labelling with ^{32}P.

Lane a: RAT1.SstI.

Lane b: RAT1 pcl-5 1L#6.SstI.

Lane c: pcl-5 1L#9.SstI.

Lane d: RAT1 pcl-5 1L#6.KpnI.

Lane e: pcl-5 1L#9.KpnI.

Lane f: RAT1 pcl-5 1L#6.BamHI.

Lane g: pcl-5 1L#9.BamHI.

Lane h: RAT1 pcl-5 1L#6.EcoRV.

Lane i: pcl-5 1L#9.EcoRV.

The numbers to the left represent molecular weight markers in kb.

Table 6. Biological activity of the molecularly cloned F-MuLV (pcl-5) in vitro

Virus	XC plaques/ml	
	A31	3T3
F4-6	5 x 10^8	6 x 10^8
RAT1 pcl-5 1LB6	3 x 10^8	3 x 10^8
643/22N	4 x 10^8	4 x 10^8

RAT1 pcl-5 1L#6 was a replication competent MuLV of NB tropism, as was also the virus being released from the uninduced cell line F4-6 (from which pcl-5 was cloned), and RAT1 643/22N (biologically cloned F-MuLV from F4-6). Thus *in vitro* the molecularly cloned F-MuLV (pcl-5) had identical biological properties as compared to its biologically uncloned and cloned counterparts.

Previous work using either the replication competent virus of the Mirand FV-P strain being released from non-induced (and indeed induced) F4-6 cells as well as the biologically cloned virus 643/22N, has shown that *in vivo* this virus is only able to cause a lymphatic leukaemia in newborn mice (Pragnell *et al*., 1978) and has thus been referred to as lymphoid leukaemia virus (also earlier by other workers, Steeves *et al*., 1971). This virus, unlike other helper virus variants in Friend virus complexes which are able to induce splenomegaly and erythroleukaemia in susceptible newborn mice (i.e. F-MuLV 57), shows no erythroid involvement in it's pathology. To test this, virus being released from the cell line RAT1 pcl-5 1L#6 was used to inoculate newborn DBA/2J mice by the intraperitoneal route. XC assay of the supernatants used revealed that 10^8 XC p.f.u. were being injected in the 100 µl inoculum. Virus being released from the cell line 643/22N was used to infect parallel groups of newborn mice as a positive control. The mice were observed for 3-4 months after which time no spleen enlargement could be detected. Animals sacrificed after this time showed no spleen or liver involvement. However greatly enlarged lymph nodes were apparent combined with an increased white blood cell count indicating that the lymphoid system had been affected in these animals, thus suggesting an abnormality in lymphoid function as predicted by previously reported data using the biologically cloned

virus and also by the identical manifestations indicated by 643/22M infected mice. Further observation of the animals up to six months revealed that the mice became sick at this time but without fatal consequences. Again no spleen involvement was observed.

v) Construction of a linear F-SFFVpM DNA molecule

As with the F-MuLV (pcl-5) described above, in order to test the biological activity of the molecularly cloned F-SFFVpM from F4-6 cells it was essential to construct a representative functional DNA genome from the fragments which had been molecularly cloned. The scheme employed is shown in Figure 38. The HindIII fragment from the plasmid p2#1 was isolated by electroelution after preparative gel electrophoresis. This fragment corresponded to the internal HindIII fragment from F-SFFVpM. Two further fragments were isolated in a similar manner from the plasmid p4#6 and corresponded to a 2.5 kb HindIII.EcoRI fragment (3' env, the LTR and all of the gag sequences to the HindIII site) and a 2.8 kb HindIII.PstI fragment (encompassing the 3' end of env the LTR and part of the gag region until the first PstI site). These three fragments were ligated together with HindIII.PstI digested pUC9. After transformation of competent NM522 bacterial cells and ampicillin selection small scale plasmid preparations were used to analyse forty eight clones. From these clones which were analysed by restriction enzyme digestion and gel electrophoresis only two proved positive in giving the correct restriction fragments expected. From one of these clones (pSM-1L) plasmid DNA was prepared by CsCl density centrifugation. Detailed restriction enzyme analysis of the clone further substantiated its identity. Thus the construction of pSM-1L meant that a colinear DNA molecule had been constructed which could be

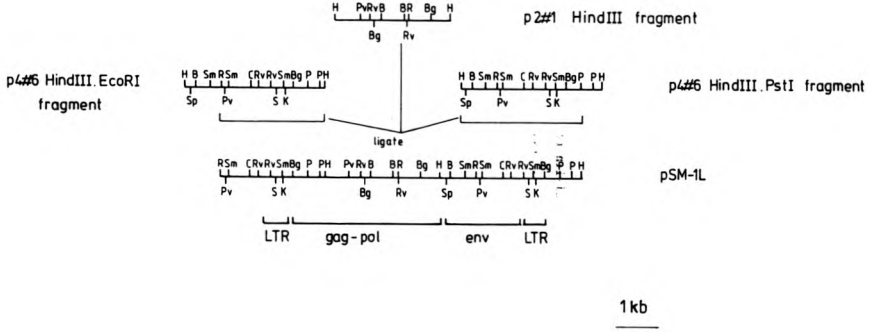

p2#1 HindIII fragment
p4#6 HindIII.EcoRI fragment
p4#6 HindIII.PstI fragment
ligate
pSM-1L
LTR
gag-pol
env
LTR
1kb

Figure 38. Construction of a linear F-SFFVpM DNA molecule (pSM-1L) from HindIII subgenomic fragments

A linear SFFVpM DNA molecule was constructed as follows: A HindIII.EcoRI fragment from p4#6 encompassing the 5' LTR and gag sequences was ligated with the p2#1 HindIII insert (the internal fragment) and a HindIII.PstI (env and 3' LTR) fragment from p4#6 and EcoRI.PstI digested pUC19. Ligation reactions were transformed into competent NM522 cells. Ampicillin resistant clones were analysed by restriction enzyme digestion for the correct construct pSM-1L.

Restriction enzyme lettering code:

B, BamHI; Bg, BglII; C, ClaI; H, HindIII; K, KpnI; P, PstI; Pv, PvuII; R, EcoRI; Rv, EcoRV; S, SstI; Sm, SmaI; Sp, SpHI; X, XbaI.

used as a substrate with which to transfect fibroblast cells.

vi) Co-transfection of RAT1 fibroblast cell with pSM-1L and a dominant selectable marker

Due to the replication defectiveness of SFFV a strategy had to be devised to detect cells that had been transfected with pSM-1L. To this end pSM-1L was co-transfected with pAG60 which is a plasmid containing the Herpes Simplex thymidine kinase promoter linked to the neomycin phosphotransferase gene. Transfection of this plasmid into eukaryotic cells confers resistance to the aminosidase analogue G418 (neomycin). Thus RAT1 fibroblasts were transfected with pSM-1L and pAG60 (at a ratio of 10:1) as in Materials and Methods. After the first medium change the medium was supplemented with G418 at a concentration of 400 µg/ml. Growth medium was changed every three days until single isolated clones could be seen (14-21 days). Ten clones were picked using cloning cylinders, expanded and genomic DNA was isolated. Southern blot analysis of SstI digested genomic DNA demonstrated that all G418 resistant clones contained several copies of pSM-1L after hybridisation with the env specific BRIE probe. In some cases recombination events had resulted in rearranged copies which did not migrate the same distance in the gel as the SstI digested pSM-1L control plasmid. Western blot analysis of three of these clones revealed that gp55 was also being synthesized in detectable amounts demonstrating that at least some of the transfected, integrated copies were capable of being transcribed into functional mRNA. One of these non-producer cell lines RAT1 pSM-1L#3 which contained no detectable rearranged copies and also synthesized detectable amounts of gp55 was then infected with F-MuLV being released from the cell line RAT1 pcl-5 1L#6, so that the

replication defective F-SFFVpM could be rescued. Supernatants from this cell line (where H stands for helper virus) were checked ten days after infection for the release of virus. In two parallel cultures of RAT1 pSM-1L#3 +H the supernatants had high reverse transcriptase titres which stayed at a high level over a period of weeks.

vii) Biological activity of SFFVpM in vivo

Using the cell lines constructed above it was intended to test whether the biological activity in vivo of the Friend virus complex reconstructed after molecular cloning and transfection (and super infection) of RAT1 cells was the same as the Friend virus complex being released from the parental cell line F4-6. To this aim groups of six DBA/2J mice were infected with five fold serial dilutions of virus being released from RAT1 pSM-1L#3 +H, F4-6 and as controls RAT1 pcl-5 IL#6 and mock infected mice (three mice per control). After eleven days the mice were sacrificed and the spleens were removed, weighed and stored in 70% ethanol.

In the positive control (F4-6) all of the mice displayed an acute splenomegaly (for undiluted and 1:5 dilutions) with numerous foci which were only quantifiable at the higher dilutions (Figure 39, Table 7). Mice infected with the FV-P complex being released from the cell line RAT1 pSM-1L#3 +H showed an identical acute splenomegaly again with numerous foci, whose number again were proportional to the dilution being inoculated. Mock infected mice (GMEM + 10% FCS) as well as F-MuLV infected mice (RAT1 pcl-5 1L#6) displayed no visible spleen enlargement.

Thus the molecularly cloned and reconstituted FV-P complex was able to induce an acute splenomegaly in susceptible mice in an identical

a b c d e

Figure 39. Splenomegaly caused by infection of DBA/2J mice with F-SFFVpM

Adult female DBA/2J mice were infected with 0.5 ml of five fold serially diluted tissue culture supernatants from F-SFFVpM producing cell lines. After 11 days the mice were sacrificed and the spleens were removed and stored in 70% ethanol after weighing.

a: control uninfected spleen.

b: F4-6 (undiluted).

c: F4-6 (1:125 dilution).

d: (undiluted).

e: (1:125 diluted).

The dilutions are shown to demonstrate confluent (b and d) and isolated foci (c and e).

Table 7. Biological activity of the molecularly cloned F-SFFVpM in vivo

Noninfected	SFFVp (F4-6) infected			SFFVp (RAT1 pSM-1L #3-H)		
Spleen wt. (g)	Virus dilution	Spleen wt. (g)	SFFU	Virus dilution	Spleen wt. (g)	SFFU
0.09 ± 0.014	undiluted	1.73 ± 0.09	n.d.	undiluted	1.8 ± 0.2	n.d.
	1:5	1.4 ± 0.2	n.d.	1:5	1.6 ± 0.1	n.d.
	1:25	0.92 ± 0.1	n.d.	1:25	1.2 ± 0.09	n.d.
	1:125	0.61 ± 0.09	n.d.	1:125	0.8 ± 0.22	n.d.
	1:625	0.32 ± 0.1	16 (18,11,18,20,14,15)	1:625	0.5 ± 0.09	80 (64,78,95,73,81,89)

DBA/2J female mice were inoculated (i.v.) with 0.5 ml virus dilutions. Eleven days post infection the mice were sacrificed and the spleens were removed, weighed and the foci were counted. Values shown represent the mean of six animals.

n.d.; not done, spleen foci too numerous to count.

The numbers in brackets represent the actual spleen foci counted at that dilution.

manner as the F4-6 FV-P complex from which the two viral components had been molecularly cloned. It was apparent from these and also the previous series of experiments using F-MuLV from RAT1 pcl-5 1L$6 that this acute splenomegaly was caused by the F-SFFVpM component of the viral complex.

The infection of susceptible mice with the FV-P complex not only leads to an acute splenomegaly after a very short incubation period, but also to a dramatic proliferation of late erythroid precursor cells (CFU-E) which are, unlike their counterparts in non-infected mice, able to proliferate in the absence of the hormone erythropoietin. This capacity is assayable in that either spleen or bone marrow cells may be plated out in methylcellulose supplemented with growth medium and CFU-E can be scored after 48 h. The presence or absence of erythropoietin in the culture medium can be used to determine whether these CFU-Es are erythropoietin independent or not.

To test this aspect and thus verify the identity of the molecularly cloned virus (F-SFFVpM), adult DBA/2J mice were infected i.v. with either FV-P being released by F4-6 or RAT1 pSM-12L$3 +H. After eleven days the mice were sacrificed, spleens were removed, weighed and single cell suspensions were plated out at various cell densities in methylcellulose growth medium supplemented with or without erythropoietin. After 48 h CFU-E were scored. Bone marrow cell suspensions were plated out similarly. The results of these experiments are shown in Table 7a.

Spleen cells from mock infected mice when plated out in the absence of erythropoietin were only (a background level is usually detectable due to low levels of epo in serum) able to give rise to very few mature CFU-E, thus demonstrating that these cells are normally dependent upon

Table 7a. Restricted biological activity of F-SFFVpM in vivo as assayed by erythroproliferative stimulation

Virus	Spleen wt. (g)	Benzidine positive CFU-E/10^8 cells			
		Spleen		Bone marrow	
		-epo	+epo	-epo	+epo
none	0.09 ± 0.04	20 ± 2	300 ± 47	30 ± 3	157 ± 61
SFFVpM (F4-6)	1.63 ± 0.1	4671 ± 221	4948 ± 188	81 ± 7	162 ± 54
SFFVpM (RAT1 pSM-1L #3+H)	1.80 ± 0.2	6512 ± 332	7439 ± 229	63 ± 7	178 ± 32

The assay for CFU-E was performed as described by Fagg and Ostertag (1982). Data are the mean of three independent experiments.

this hormone. This also shows that the components used in this assay (notably the calf serum) contained no substantial erythropoietin like activity. However in the presence of erythropoietin CFU-Es were readily detected in the spleen.

In F4-6 infected spleens there were two dramatic observations to be seen in that there was an enormous expansion in the number of CFU-E present which at the same time were erythropoietin independent, a characteristic expected for SFFVp. Infection of mice with the FV-P complex being released from the RAT1 pSM-1L#J +H cell line resulted in an identical picture as seen for F4-6 in that the spleens demonstrated an enormous proliferation of CFU-Es which were erythropoietin independent. Thus in the spleen at least the SFFVp being released from the cell lines F4-6 and RAT1 pSM-1L#J +H were identical.

As mentioned in the introduction, most SFFVp variants induce a large increase of erythropoietin independent CFU-Es in the spleen, but have very little effect upon CFU-E formation in the bone marrow. However it has been reported that the FV complex released by some Friend cells (notably the Mirand strain of SFFV used in this laboratory) is able to cause a profound erythropoietic proliferation in the bone marrow (CFU-E) in susceptible mice (Fagg et al., 1980; Fagg and Ostertag, 1982). Thus bone marrow cells of the above infected mice were plated out at various cell densities in methylcellulose as for the spleen cells and CFU-E were scored after 48 h (Table 7a).

In non-infected control mice, in the absence of erythropoietin a background level of CFU-E comparable to that seen in the spleen could be detected. In the presence of erythropoietin CFU-E could be detected although not at the level seen in the spleen. Mice infected with the FV colmplex being released from F4-6 surprisingly showed very

few erythropoietin independent CFU-E although with erythropoietin comparable numbers as seen in the control could be scored. A similar picture was seen with RAT1 pSM-1L#3 +H infected mice although the number of erythropoietin independent CFU-E was approximately two-fold higher, this was still not within the range which has been reported by Fagg and Ostertag (1982). This result is somewhat puzzling although similar observations to those reported here have been repeatedly found by other members of this laboratory (T. Franz, pers. commun.). Why these results of Fagg and Ostertag (1982) are not reproducible remains unclear although one possibility may be that the culture conditions (such as serum batches etc.) are somehow inhibitory to the formation of CFU-E in bone marrow cultures, as difficulties in the bone marrow CFU-E assay have been encountered in general (C.Laker, G.Gränning, pers. commun.).

Discussion

The molecular cloning of both components of a Friend virus complex has been described. In the procedure adopted the extrachromosomal DNAs corresponding to circular proviral intermediate molecules found within the Friend cell line F4-6 after DMSO induction were molecularly cloned either in a plasmid or a bacteriophage cloning vector. Although the F-MuLV was readily cloned in the plasmid vector pUC19 no intact SFFVp molecules could be obtained by this method. From the library screened all of the SFFVp related molecules examined had undergone deletions. What was further surprising was that all of the deletions, without exception, encompassed the *env* region of SFFVp. From these observations it may be proposed that the combination of cloning site (*Sph*I) and

cloning vector (together with the recipient bacteria) somehow led to the alterations. The SphI site is positioned 5' to the splice acceptor site for the env gene (as determined from sequence analysis of other cloned SFFVs) thus digestion with this enzyme would leave the env gene intact. Cloning within an expression vector such as pUC19 could thus result in transcription and translation of the env gene product which could be envisaged to have deleterious effects on the bacterial life cycle, for not all eukaryotic proteins are expressable in certain strains of bacteria. However even the one clone which was obtained with the pBR322, DH1 combination had a rearranged env gene. In this cloning vector the SphI site is within the tetracycline resistance gene approximately 600 bp downstream from the promoter. Thus for the env product (assuming to be in the correct orientation) to be synthesized, either it must be a fusion protein or initiated from internal sequences. Both of these are unlikely so one could propose that the env sequences per se were poisonous and thus led to the recombinants observed.

These problems for SFFVp were overcome by cloning the virus in two fragments in a bacteriophage cloning vector in which digestion with the restriction enzyme HindIII disrupted the continuity of the env gene. This approach proved more successful allowing the molecular cloning of the Mirand strain of SFFVp and subsequent construction of a detailed restriction enzyme map.

When one compares the biological activity of pcl-5 with F-MuLV 57 then pcl-5 causes a lymphatic leukaemia whereas F-MuLV 57 is able to induce an erythroleukaemia in newborn mice. It has been shown that the disease specificity of the helper virus appears to be determined by the viral LTR (Bősze et al., 1986). Recombinants between F-MuLV and Mo-MuLV

as well as between F-MuLV and amphotrophic MuLV constructed by exchanging fragments containing the 3' end of the genomes demonstrated that the LTR was responsible for the tropism of the viruses (Chatis et al., 1983, 1984; Des Groseiller and Jolicoeur, 1984; Des Groseiller et al., 1983; Oliff et al., 1984; Holland et al., 1985, 1987). One interesting point is that it has also been shown that leukaemia induced by F-MuLV is also dependent upon gag sequences (Oliff et all., 1985).

The genome size of both the anaemia and polcythaemia inducing SFFVs is remarkably variable. This probably reflects their long evolutionary history in different laboratories and also indicates that large parts of the genome are not essential for the rapid induction of erythroleukaemia in adult animals. Thus when the F-SFFVpM is compared with the other molecularly cloned SFFVs (Figure 34) one can see that although some restriction enzyme sites are common to most of the SFFVs (notably in the env region) there is no extensive homology at this level. The size heterogeneity of the different SFFVs is mainly due to deletions within the gag-pol region as shown by immune precipitation of viral proteins, oligonucleotide fingerprints and hybridisation experiments (Bilello et al., 1980; Mol et al., 1982; Langdon et al., 1983; Ruscetti et al., 1984).

As previously mentioned it has been demonstrated with the Mirand strain of SFFVp that upon induction of various Friend cell lines (transformed by the Mirand strain, including F4-6) that a novel SFFV is synthesized (as shown at the RNA level) and indeed released presenting a biological activity, erythroid proliferation, extending to the bone marrow (Ostertag and Pragnell, 1981). In these studies it was suggested that the DMSO induced differentiation of F4-6 led to the synthesis and release of an "original" SFFV as well as an N-tropic helper virus. Thus

it was hoped that in the analysis presented here and earlier more light could be shed onto this phenomenon in that this new SFFVp could be further characterised at the molecular level and also possibly molecularly cloned. However in the primary analysis no detectable heterogeneity in the size of SFFVp related extrachromosomal DNA molecules was seen during the course of induction and indeed upon bulk preparation and purification thus suggesting the presence of a single DNA species (so far as could be determined by this analysis for no aberrant bands were detected after restriction enzyme mapping). That after the molecular cloning of this SFFV DNA and subsequent biological testing proved that the cloned F-SFFVpM had a biological activity restricted to the spleen with very little effect on the bone marrow, suggested that either the novel SFFV was not present as an extrachromosomal DNA form within these cells or was not molecularly clonable using the strategy presented.

Chapter 3

Construction of a biologically active F-SFFVp retroviral vector containing a dominant selectable marker gene

Introduction

It has been generally realised for some time that retroviral genomes are good candidates for vehicles to introduce desirable genes into eukaryotic cells (Shimotohno and Temin, 1981; Tabin et al., 1982; Williams et al., 1985). This has been greatly aided by an intimate understanding of the functional organisation of retroviral genomes and a full appreciation of the large variety of ways in which retroviruses have acquired onc genes as part of their genetic make up. There are a number of characteristics of the retrovirus life cycle that in combination render these agents uniquely suited for certain types of experimental manipulations involving expression of foreign genes in cells and in whole animals. Their ability to efficiently integrate into the host chromosomal DNA and be stably transmitted as well as the broad spectrum of cells which they are able to infect makes retroviruses a more attractive and superior gene transfer system than calcium phosphate transfection (Wigler et al., 1977; Wigler et al., 1978).

It has been shown that virally transduced genes are expressed at 10-50 fold higher levels than those introduced by DNA mediated gene transfer (Hwang and Gilboa, 1984). Thus much effort has been put into developing retrovirus vectors. The introduction of genetic markers into the retrovirus genome has greatly extended the utility of these vectors for selecting and identifying infected cells (Coffin, 1985). The introduced marker gene may either be under control of an independent promoter, which may be their own, or of those promoter/enhancer elements found within the LTRs of the retrovirus.

Attempts to construct a biologically active SFFV containing a dominant selectable marker gene have until now met with limited success

(Joyner and Bernstein, 1983; Hess, 1985). In both cases deletions after transfection of the viral genomes gave rise to recombinant viruses in which the selectable marker and transforming functions had segregated. However other selectable, biologically active retroviruses have been successfully constructed (Wei et al., 1981; Ostertag et al., 1986; Friel et al., 1987).

Little is known about the target cell interactions that lead to erythropoietin independent differentiation of SFFVp infected erythroid precursor cells. In order to study these it is necessary to define the target cells. A study of target cell/SFFV interaction has proven to be difficult, even though the efforts of Hankins group on in vitro infection in mass cultures have confirmed many of the assumed properties of the SFFV target cells (Hankins and Troxler, 1980). The exact target cell of SFFV however remained elusive. Differentiation induction and/or malignant transformation mediated by SFFV has also not been achieved with sorted and enriched erythroid precursor cells (Johnson and Ostertag, pers. commun.).

The main problem to define the target cell(s) has been on the one hand the in vitro cell system and on the other hand the problems involved in tracing infections of SFFV to single cell progeny. Better cell systems are now available with stem cell lines that can be induced to differentiate along any of the myeloid lineages including the erythroid pathway (Boettiger et al., 1984; Spooncer et al., 1986).

A selectable marker gene within the frame work of a biologically active SFFV would also be of advantage in analysing the causes which may lead to malignancy of Friend cells, i.e. aiding analysis of the integration site(s) of SFFV. Finally the intriguing correlation between induction of viral functions and erythroid differentiation (Ostertag et

al., 1987) of Friend cells could be studied more easily if SFFV genomes could be assayed not only by in vivo assays but also by in vitro assays utilising a selectable marker gene.

In this chapter the following questions were posed.

1) Is it possible to avoid the difficulties in constructing a hybrid selectable SFFVp?

2) Is it possible to elicit an erythroid hyperprofilerative disease with such a recombinant neo^R SFFVp?

3) Can malignant Friend type cells be generated with such a recombinant vector virus?

A brief overview of the MPSV biology and molecular biology will be presented here as a prior knowledge for the construction of neo^R SFFV.

The myeloproliferative sarcoma virus (MPSV) is a member of the Moloney murine sarcoma virus (Mo-MuSV) family. MPSV is unique for not only does it efficiently transform fibroblasts in vitro and cause sarcomas upon intramuscular inoculation of adult mice, a property also of Mo-MuSV, but it also gives rise to extensive haemopoietic changes in vivo characterised by proliferation of late erythroid precursor cells, haemopoietic stem cells and granulocyte-macrophage precursor cells (Ostertag et al., 1980; Klein et al., 1981). Molecular analysis of the MPSV genome has revealed that it is composed entirely of Mo-MuLV and cellular mos proto-oncogene derived sequences (c-mos), as is the Mo-MuSV (Kollek et al., 1984). It has been shown that the unique leukaemia inducing capacity of MPSV is due to a cooperative interaction between the U3 region of the 5' LTR, which differs slightly from those of the related Mo-MuLV and MuSV, and the mos oncogene (Stacey et al., 1984; Stocking et al., 1985; Stocking et al., 1986; Ostertag et al., 1987). MPSV is like most other oncogenic viruses replication defective.

Results

a) Construction of the neo^R SFFVp retroviral vector

It was intended to construct a neo^R SFFVp vector using the SFFVp cloned in the previous chapter, however due to the problems encountered with the molecular cloning of this viral genome, it was decided to utilise a subgenomic fragment from the Lilly and Steeves strain of SFFVpLS (Linemeyer et al., 1980), in the construction of this vector. Further arguments which substantiated this decision, were that construction of such a retroviral vector requires, nucleotide sequence data, so that various important sequences (i.e. splice acceptor sites and restriction enzyme sites) can be recognised and thus utilised in the construction process, and various derivatives thereof. It was thus decided to use the SFFVpLS clone, which is indistinguishable in its biological activity, from F-SFFVpM.

The efforts of Joyner and Bernstein (1983) and Hess (1985) to construct a selectable SFFV, utilised the genome of SFFVp as a backbone, and attempted to insert the marker within the non-functional gag-pol region. In the construction of neo^R SFFVp a different approach was used, in that a hybrid virus consisting of the 5' LTR, gag, 3' pol, and the neomycin resistance gene sequences, originating from the neo^R MPSV (neo^R mos^{-R}), together with the env gene and 3' LTR from SFFVpLS, was constructed (Figure 40).

A 2.5 kb HindIII-PstI fragment encoding the env gp55 gene with its 3' splice acceptor sequences, a complete 3' LTR and part of the gag sequence, was isolated from the pSFFVpLS plasmid, by preparative agarose gel electrophoresis and electroelution. A similar procedure was used to isolate a 6 kb EcoRI-HindIII fragment from the neo^R mos^{-R}

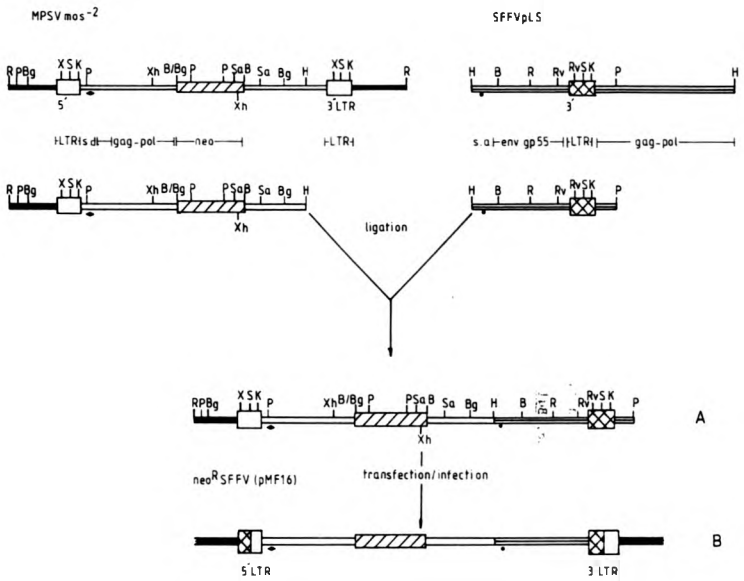

MPSV mos -2
SFFV pLS
R P Bg X S K P Xh B/Bg P P Sa B Sa Bg H X S K R
5'
Xh
3' LTR
H B R Rv Rv S K P H
3'
5' LTR gag-pol neo 3' LTR
5' LTR s.a. env gp55 LTR gag-pol
ligation
transfection/infection
neo^R SFFV (pMF16)
A
B
5' LTR
3 LTR

Figure 40. Construction of neo^R SFFVp

neo^R SFFVp was constructed as described in the text. Transformation of competent CMK603 followed by small scale plasmid DNA analysis, led to the isolation of a clone pMF16, the structure of which is shown (cloned into the EcoRI and PstI sites of pUC19). See A.

The restriction enzyme code is as follows

B, BamHI; Bg, BglII; H, HindIII; K, KpnI; P, PstI; R, EcoRI; Rv, EcoRV; S, SstI; Sa, SalI; X, XbaI; Xh, XhoII.

◆ s.d. splice donor sequence.

● s.a. splice acceptor sequence.

B) This diagram illustrates that after one round of viral replication i.e. reverse transcription, the U3 region of the SFFV 3' LTR will also be present in the 5' LTR.

The shaded areas represent genomic DNA sequences either cloned with and flanking the integrated MPSV provirus, or genomic flanking sequences after integration of neo^R SFFVp.

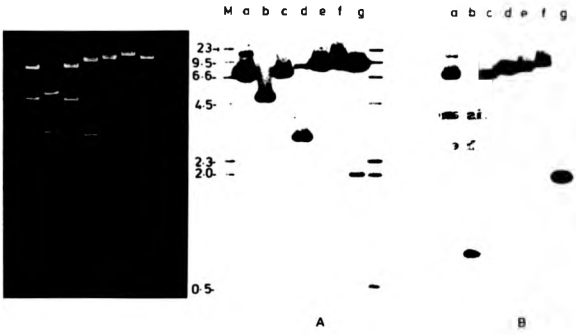
plasmid. This fragment contains cellular genomic DNA sequences 5' to the LTR (as a consequence of cloning the integrated proviral MPSV DNA), gag and pol sequences, as well as the Tn5 neomycin resistance gene.

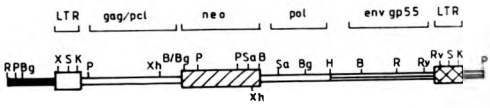
Neo^R SFFVp was constructed by ligating these two fragments (in equimolar concentrations of approximately 200 ng) together with EcoRI-PstI digested pUC19 (100 ng) as described in Materials and Methods. Recombinant plasmids were screened by ampicillin resistance selection after transformation of competent CMK603 bacterial cells. Analyses of twenty four clones, revealed that four had the predicted restriction enzyme pattern after digestion with PstI. One clone (pMF16) was then grown up for large scale plasmid preparation and further analysed by restriction enzyme analysis combined with Southern blotting. The results of this analysis are shown in Figure 41.

From this analysis it can be seen that the predicted restriction enzyme map is correct giving the correct restriction enzyme fragments after digestion with SstI, PstI, EcoRI, HindIII, BamHI, SalI, and XhoI. That these fragments also correspond to the appropriate genes was shown in that hybridisation with both neo and env (BRIE) specific probes gave rise to the expected hybridisation pattern (Figure 41).

b) Construction of neo^R SFFVp non-producer and producer cell lines

The neo^R SFFVp vector described above has a 5' LTR originating from MPSV and a 3' LTR from SFFVp. However, due to the replication strategy, after one round of infection and replication, both the 5' and 3' LTRs will have U3 regions of SFFVp origin (Figure 40). Since these regions contain the transcription control elements for the retrovirus, the ensuing virus will be regulated by SFFVp specific U3 control sequences. Thus in order to obtain neo^R SFFVp non-producer and producer cell





1Kb

NEO^{R}SFFV

Figure 41. Restriction enzyme analysis of pMF16 (neo^R SFFV).

Plasmid DNA (0.5 µg) of pMF16 (neo^R SFFVp in pUC19) was digested to completion with the enzymes SstI (lane a), PstI (lane b), EcoRI (lane c), HindIII (lane d), BamHI (lane e), SalI (lane f) and XhoI (lane g). After separation of the fragments by agarose gel electrophoresis and subsequent Southern blotting the filter was hybridised with either the neo (B) or gp55 env specific probe BRIE (A).

A photograph of the ethidium bromide stained gel is depicted to the left (P).
Also shown under the figure are the origin of the probes used in this and further analysis using neo^R SFFV and related vectors.

The numbers to the left of A represent molecular weight markers in kb, lane M.

lines a strategy was used, to ensure that one round of replication had been completed before isolating the relevant cells. This was performed as follows. Neo^R SFFVp DNA (pMF16, 5 µg/T75 flask, i.e. 1 x 10^6 cells) was transfected into both PA317 and psi-2 cells by the calcium phosphate precipitation method. These two fibroblast cell lines have the capacity to encapsidate defective retroviral RNA genomes, and thus release, helper free infectious virus. Viruses released from PA317 have an amphotropic host range, whereas viruses released from psi-2 virus have an ecotropic host range.

The cells were cultured for 48 h in GMEM + 10% FCS at 37°C and 5% CO_2. After this period of time, the supernatants from these cell cultures, were filtered (0.45 µm) before being used to infect either PA317 cells (psi-2 supernatant), psi-2 cells (PA317 supernatant) or RAT1 fibroblasts (both psi-2 and PA317 supernatants). These infected cells were then selected for successful viral transfer (G418 resistance) by incubation in medium supplemented with G418 at a concentration of 400 µg/ml. G418 resistant clones appeared after 14-20 days and were picked and expanded for further analysis.

Supernatants of the resulting psi-2 and PA317 clones were assayed for reverse transcriptase, to detect quantitatively virus being released from these cells. Only those cells releasing appreciable amounts of virus (i.e. >50% of the positive control) were kept for further analysis.

i) Southern blot analysis of neo^R SFFVp infected cell lines

High molecular weight cellular genomic DNA was isolated from G418 resistant, psi-2, PA317 and RAT1 clones, and was analysed by Southern blot hybridisation following digestion with restriction enzymes to

confirm integrity, stability and copy number of the viral genomes (Only the data for the RAT1 clones are shown. Figure 42).

Digestion with the restriction enzyme SstI which cuts once within the LTR of the viral genome gave rise to a fragment of 8 kb which hybridised both to neo and SFFVp env (BRIE) specific probes.

Most of the integrated copies were of the correct size (as compared to the SstI digested plasmid pMF16, which was run as a control). However, there were also cell lines which contained altered integrated viral DNA copies, mostly with deletions, which had probably arisen as a product of the transfection and recombination events leading to aberrant proviruses (Figure 42). From this analysis, five cell lines from each group (i.e. RAT1, psi-2 or PA317), containing the correct sized genome were catalogued frozen and stored in liquid nitrogen. The remaining cell lines were discarded.

ii) Expression of neo^R SFFVp functions in fibroblasts

RNA isolated from neo^R SFFVp fibroblasts (PA317, psi-2 and RAT1) was analysed to confirm the correct transcription and processing of the neo^R SFFVp construct.

PA317 and psi-2 cell lines having high reverse transcription titres were analysed (clones PA317 neo^R SFFV #5, psi-2 neo^R SFFVp #11 and psi-2 neo^R SFFVp #6). From this analysis it can be seen that both full genomic viral RNA (appr. 8 kb, seen upon hybridisation with env) as well as spliced env specific subgenomic RNA were detected in the northern blots shown in Figure 43 (Bilello et al., 1980). It can be seen however that one of the RAT1 non-producer clones, (RAT1 neo^R SFFVp #8a) synthesized considerable amounts of genomic and subgenomic RNA. Due to this observation and also to the nature of the experiments

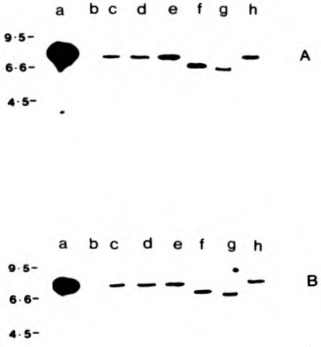

a b c d e f g h
9·5-
6·6-
4·5-
A
a b c d e f g h
9·5-
6·6-
4·5-
B

Figure 42. Southern blot analysis of neo^R SFFVp infected RAT1 clones

Genomic DNA (10 µg) was digested to completion with SstI before being subjected to agarose gel electrophoresis and Southern blotting. The filter was hybridised with either a neo (A) or an env specific (BRIE) ^{32}P labelled probe (B).

Lane a: neo^R SFFVp plasmid DNA (pMF16, 80 pg).

Lane b: RAT1.

Lane c: RAT1 neo^R SFFVp #1a.

Lane d: RAT1 neo^R SFFVp #2.

Lane e: RAT1 neo^R SFFVp #4.

Lane f: RAT1 neo^R SFFVp #5.

Lane g: RAT1 neo^R SFFVp #6.

Lane h: RAT1 neo^R SFFVp #8a.

The numbers to the left represent molecular weight markers in kb, lane M.

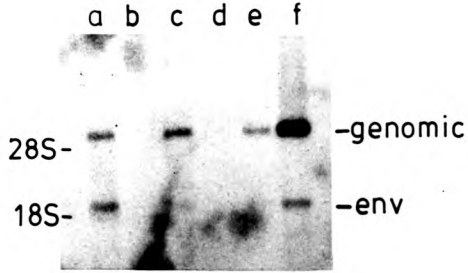

a b c d e f
28S-
18S-
-genomic
-env

Figure 41. Nothern blot analysis of neo^R SFFVp infected producer and non-producer cell lines

In this analysis 1 µg of polyA$^+$ RNA from each of the cell lines was electrophoresed on a 1.0% MOPS gel as described in Materials and Methods. After blotting onto Gene Screen Plus the RNA was hybridised with an *env* (BRIE) specific probe, which detects both genomic mRNA as well as the spliced *env* mRNA.

Lane a: F4-6.

Lane b: RAT1.

Lane c: PAJ17 neo^R SFFVp #5.

Lane d: psi-2 neo^R SFFVp #11.

Lane e: psi-2 neo^R SFFVp #6.

Lane f: RAT1 neo^R SFFVp #8a.

After removing the *env* probe, and hybridising with a neo specific probe only the genomic band was detected (data not shown).

planned, (see later) most of the effort was concentrated on the RAT1 non-producer cell line RAT1 neo^R SFFVp #8a, however, where parallel experiments were performed with PA317 or psi-2 clones, the data will be presented.

iii) Co-expression of the selectable marker and of the env related gp55 gene in fibroblasts

Co-expression of both genes, the selectable marker gene and the env gp55 gene was examined in order to exclude cross-interference of virally coded functions as has been reported for other vectors (Bandyopadhyay and Temin, 1984; Emerman and Temin, 1984).

In addition it was desirable to generate a neo^R SFFVp producer cell line which could be utilised as a source of virus for infection of mice in order to establish the pathogenicity of the neo^R SFFVp construct.

The non-producer RAT1 cell clone #8a (which contains a single intact vector genome, which is transcribed) was infected with molecularly cloned F-MuLV virus (RAT1, pcl-5 1L#6), as a helper virus. At time intervals of three days, supernatants were removed from the RAT1 neo^R SFFVp #8a+H (where H stands for helper virus) cell culture and assayed for reverse transcriptase activity. After ten days, this cell line was then tested for the titre of neo^R SFFVp virus being released, in the Geneticin transfer assay. The results of these experiments are depicted in Table 8, together with the titres being released from psi-2 and PA317 clones, having the highest reverse transcriptase titres.

From this analysis it can be seen that, the RAT1 neo^R SFFVp #8a+H cell line releases high titres of neo^R SFFVp (1.5×10^6 GTU/ml) whereas the psi-2 and PA317 cell lines released only moderate titres (1×10^5 -

Table 8. GTU titres released by neo^R SFFVp infected cell lines

Cell Line	GTU/ml being released into tissue culture supernatant*
RAT1 neo^R SFFVp #8a+H	1.5×10^4
psi-2 neo^R SFFVp #1	1×10^3
psi-2 neo^R SFFVp #6	5×10^4
psi-2 neo^R SFFVp #7	2×10^3
psi-2 neo^R SFFVp #11	3×10^3
psi-2 neo^R SFFVp #17	1×10^3
PA317 neo^R SFFVp #5	2×10^3
PA317 neo^R SFFVp #9	2×10^4

* The GTU assay was performed on RAT1 fibroblasts as described in Materials and Methods.
Results represent the mean of dilutions performed in triplicate.

5 x 10^4 GTU/ml).

These G418 resistant cell lines were then tested for expression of the env glycoprotein gp55. ^{35}S-methionine labelled cell lysates were immune precipitated with a goat anti-Friend virus antiserum. The Friend cell line F4-6 which releases high titres of F-SFFVpM was used as a positive control, along with RAT1 pcl-5 1L#6, which is a F-MuLV producer cell line (Figure 44). In this analysis it can be seen that the positive control, F4-6 (lane c) synthesized high levels of gp55 (as well as gp70 and Pr85). This is not unexpected, for SFFV infected Friend cells are known to synthesize gp55 at much higher levels than SFFV infected fibroblasts (Bilello et al., 1980). Considerable amounts of gp55 are also present in the RAT1 neo^R SFFVp cell lines (#8a, lane b and #8a+H, lane j). Also seen in the RAT1 neo^R SFFVp #8a+H cell line are the F-MuLV specific gp70 and Pr85 env gene products, which are also seen in the RAT1 pcl-5 1L#6 (F-MuLV infected) cell line (lane d).

The psi-2 cell lines analysed here, synthesize barely detectable amounts of gp55, with psi-2 neo^R SFFVp #6 producing the largest amounts (lane f). The gp70 molecules seen in the psi-2 cell lines originate from the defective Mo-MuLV genome integrated in the cell lines.

iv) neo^R SFFVp is stably transferred in vitro

Although the cell line RAT1 neo^R SFFVp #8a+H contains a single stably integrated proviral genome, which is transcribed to give the correct gene products (i.e. G418 resistance and gp55 synthesis) and also releases virus which is able to confer G418 resistance to infected cells (as seen from the GTU titres), it was important to see whether the virus being transferred was stable. To test this, virus released by the cell line RAT1 neo^R SFFVp #8a+H was used to reinfect RAT1 cells to

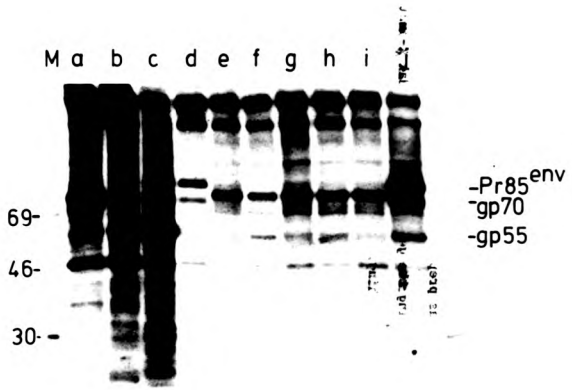

M a b c d e f g h i
69-
46-
30-
-Pr85env
-gp70
-gp55

Figure 44. Immune precipitation analysis of neo^R SFFVp infected cell lines. Dual expression of G418 resistance and gp55.

Cell lysates from ^{35}S labelled cells (3 h labelling), were immune precipitated with a goat anti-Friend virus antiserum (provided by P. Nobis, University of Hamburg). The precipitated proteins were then resolved on a 10-30% SDS gradient gel, which after completion of electrophoresis, was prepared for fluorography as in Materials and Methods. After drying the gel was exposed to Kodak X-omat A film at -70°C, after pre-flashing.

Lane a: psi-2

Lane b: RAT1 neo^R SFFVp #8a

Lane c: F4-6

Lane d: RAT1 pcl-5 1L#6

Lane e: psi-2 neo^R SFFVp #1

Lane f: psi-2 neo^R SFFVp #6

Lane g: psi-2 neo^R SFFVp #7

Lane h: psi-2 neo^R SFFVp #11

Lane i: psi-2 neo^R SFFVp #17

Lane j: RAT1 neo^R SFFVp #8a+H

The numbers to the left represent protein molecular weight markers in kd, lane M.

obtain a series of secondarily infected (and G418 selected, 400 µg/ml) neo^R SFFVp cell lines at end point dilution. The neo^R SFFVp virus integrated proviral DNA genome was then analysed in 25 of these clones by Southern blotting to test the stability of the neo^R SFFVp on viral transfer. Digestion of these DNAs with SstI and hybridisation with the neo probe, showed that all viruses were of the correct size (data for 14 of these clones are shown in Figure 45). All of these clones also showed expression of gp55 as determined by western blot analysis, (data not shown).

c) Neo^R SFFVp induces an acute erythroid hyperplasia identical to that caused by wild type SFFVp

All previously tested selectable SFFV vectors had grossly altered biological properties when tested in vivo (Joyner and Bernstein, 1983; Hess, 1985). The genome of virus rescued after such infections was rearranged and as a consequence of that the virus no longer dually expressed the selectable marker gene and transforming functions. Thus to analyse this aspect and also to see whether the neo^R SFFVp could induce a disease which was indistinguishable from the wild type SFFV, susceptible mice were infected with virus (neo^R SFFVp+ F-MuLV) being released from the cell line RAT1 neo^R SFFVp #8a+H. Filtered supernatants (0.45 µm) containing virus were injected intravenously in serial 5 fold dilutions into groups of six DBA/2J female mice. These virus dilutions were also titrated in vitro in the GTU assay to determine the number of viable (i.e. conferring G418 resistance to infected cells) neo^R SFFVp virions being transferred.

The prototype SFFV of the Friend cell line F4-6 was injected as a positive control. Eleven days p.i., the mice were sacrificed, spleens

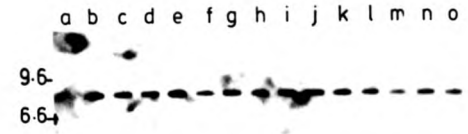

a b c d e f g h i j k l m n o
9·6
6·6

Figure 45. Southern blot analysis of G418 resistant RAT1 clones infected with virus released from RAT1 neo^R SFFVp #8a-H. neo^R SFFVp is stably transferred.

High molecular weight genomic DNA (10 µg) from individual G418 resistant RAT1 clones was digested with SstI. After electrophoretic separation of the digested DNA fragments and subsequent Southern blotting the filter bound DNA was hybridised with a ^{32}P radiolabelled neo specific probe.
The analysis of 14 of the 25 clones are shown below although the remaining 11 clones displayed identical results.

Lane a: RAT1 neo^R SFFVp #8a-H.

Lane b: to lane o: neo^R SFFVp infected RAT1 clones.

The numbers to the left represent the molecular weight markers in kb

were removed, weighed and fixed in 70% ethanol, prior to the counting of foci. All mice infected with neo^R SFFVp showed grossly enlarged spleens with numerous foci (Figure 46; Table 9). Comparison, with the unmodified SFFV being released from F4-6, show that neo^R SFFVp induces pathological symptomes indistinguishable from those of the wild type SFFV. Indeed histological examination of both neo^R SFFVp and wild type SFFV infected spleens displayed identical features, mainly massive infiltration of erythroblasts into the red pulp of diseased spleens (Figure 47) as is typical for mice infected with SFFVp.

d) neo^R SFFVp infected erythroid precursor cells are increased, do not require erythropoietin for colony formation and are resistant to geneticin (G418)

It has already been shown, that in neo^R SFFVp infected fibroblasts there was co-expression of G418 resistance and synthesis of SFFV coded gp55. It was thus important to obtain evidence for co-expression of SFFV env transforming properties and geneticin resistance in erythroid target cells, in vivo. Two transformation properties can easily be tested in neo^R SFFVp infected mice. Expansion of erythroid precursor cells and erythroid proliferation in the absence of the normally required hormone erythropoietin. Erythroid colony formation in vitro of in vivo infected spleen cells can be utilised to test for these properties.

Adult DBA/2J female mice were inoculated either with 5 x 10^3 SFFU/ml prototype SFFV supernatant (F4-6) as a positive control or 2 x 10^8 GTU equivalent of neo^R SFFVp. After eleven days p.i. mice were sacrificed, spleens removed and cell homogenates were plated out at various densities in methylcellulose in the presence or absence of

a b c d e

Figure 46. neo^R SFFVp is able to cause an acute splenomegaly with discrete spleen foci in susceptible mice

Eight week old DBA/2J female mice were inoculated (i.v.) with either FV-P being released from F4-6 (5000 SFFU) or neo^R SFFVp being released from RAT1 neo^R SFFVp #8a+H (1 x 10^6 GTU).
Eleven days post infection mice were sacrificed and the spleens were removed, and stored in 70% ethanol.

a) Uninfected spleen.

b) F4-6 wild type SFFVp (1:125 diluted).

c) F4-6 wild type SFFVp (undiluted).

d) neo^R SFFVp (1:125 diluted).

e) neo^R SFFVp (undiluted).

Table 9. Neo^R SFFV$_p$ induces acute splenomegaly and foci in susceptible mice

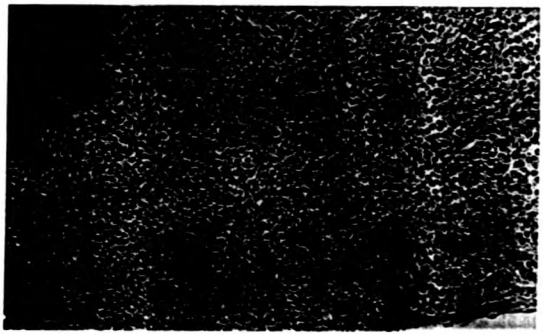
Noninfected	SFFV$_p$ (F4-6) infected			Neo^R SFFV$_p$ (8a+H) infected				
Spleen wt. (g)	Virus dilution	Spleen wt. (g)	SFFU[a]	Virus dilution	Spleen wt. (g)	GTU[b]	SFFU	SFFU/GTU
0.096 ± 0.014	Undiluted	1.625 ± 0.2	n.d.	Undiluted	1.78 ± 0.15	1.56×10^6	n.d.	-
	1:5	1.325 ± 0.1	n.d.	1:5	1.39 ± 0.2	3.12×10^5	n.d.	-
	1:25	0.76 ± 0.11	n.d.	1:25	0.62 ± 0.09	6.25×10^4	n.d.	-
	1:125	0.61 ± 0.09	n.d.	1:125	0.46 ± 0.1	1.25×10^4	n.d.	-
	1:625	0.35 ± 0.09	30 (34,27,35,29,25,30)	1:625	0.27 ± 0.06	2.50×10^3	n.d.	-
				1:3125	0.11 ± 0.07	5.00×10^2	40 (35,31,47,41,47,39)	0.08

DBA/2J mice were inoculated (i.v.) with 0.5 ml dilutions of either SFFVp or neo^R SFFV$_p$. Eleven days post infection the mice were sacrificed and the spleens were removed, weighed, fixed, and the foci were counted (a). For neo^R SFFV$_p$ the virus dilutions injected were also titrated to end point in vitro in the RAT1 GTU assay (b).

Values shown represent the mean of six animals. The numbers in brackets represent actual spleen foci counted at that dilution.

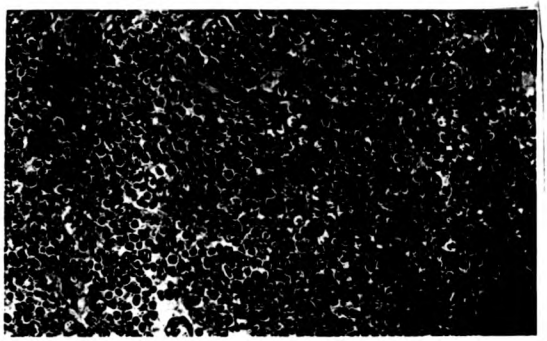
n.d.; not done, foci too numerous to count

A

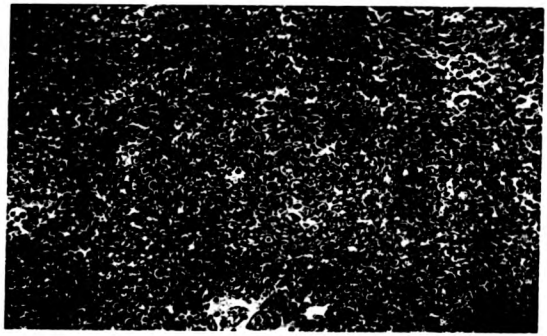


B

C



D



E

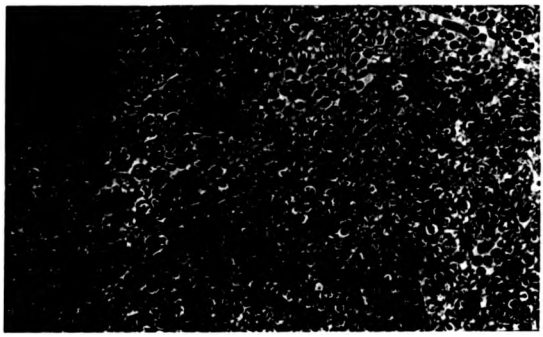


Figure 47. Histopathological analysis of spleens from neo^R SFFVp infected mice

All sections were prepared as outlined in Materials and Methods and after staining were photographed at either 25x or 40x magnification.

A. Uninfected spleen (25x). A section showing the red pulp of the spleen in which very few large blast cells can be seen. Areas of active erythropoiesis can be seen (E) which give rise to fully differentiated blood cells (erythrocytes, ery).

B. F4-6 infected spleen eleven days post infection (25x). The section demonstrates the presence of large numbers of erythroid blasts (Eb) and normocytes (N). Very little fully differentiated erythropoiesis is seen.

C. F4-6 infected spleen eleven days post infection (40x). The heavily staining cells represent normocytes (N) and the larger cells represent erythroid blasts (EB).

D. neo^R SFFVp infected spleen eleven days post infection (25x). This picture shows a similar pattern as shown in B.

E. neo^R SFFVp infected spleen eleven days post infection (40x).

erythropoietin and G418 and erythroid colonies (CFU-E) were counted after 48 h (Table 10).

CFU-E in the spleens of mock infected mice required, as expected, the presence of exogenously added erythropoietin. Addition of G418 (1.5 mg/ml) to the culture medium inhibited the formation of these CFU-E colonies. Those few colonies that survived were smaller than regular CFU-E with only a small fraction of these colonies being benzidine positive (B^+) (Figure 48). 1.5 mg/ml G418 thus inhibited, within two days the development of late erythroid colonies. This is an observation that is not entirely unexpected if toxicity of geneticin is related to active metabolism and/or cell division. Late erythroid progenitors are known to be fast replicating cells, passing through an average of 7 cell divisions within two days to generate colonies of more than 100 cells.

Infection of mice with wild type SFFVp (F4-6) yielded an enormous increase in the number of CFU-Es which proliferated in the absence of exogenously added erythropoietin as expected. The number of CFU-Es scored either in the presence or absence of erythropoietin was always significantly lower when cultured in the presence of G418. Almost all of the CFU-Es were benzidine positive (B^+) if no G418 was added, however in the presence of 1.5 mg/ml G418, colonies were much smaller than those of uninfected controls. They did not differentiate to synthesize haemoglobin and were thus benzidine negative (B^-), again like G418 treated control CFU-E.

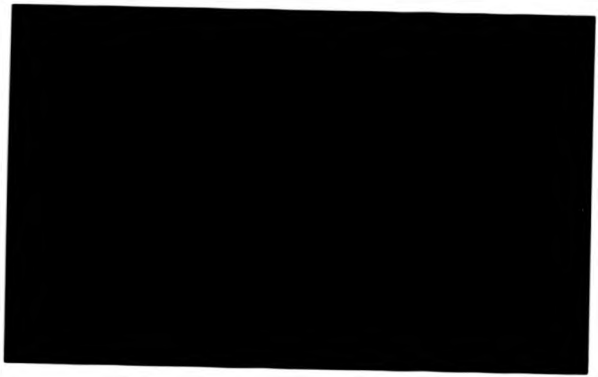
Infection of mice with neo^R SFFVp resulted in an enormous increase of late erythroid precursor cells in the spleens of infected mice. These CFU-E did not require erythropoietin. CFU-Es of these infected animals, in contrast to controls were resistant to G418 and gave rise to large

Table 10. Spleen erythroid precursor cells of mice infected with neo^R SFFV$_p$ are increased, erythropoietin independent and resistant to genecitin

Virus	Spleen wt. (g)	Benzidine positive CFU-E/10^5 spleen cells			
		(-) Epo		(+) Epo	
		-G418	+G418	-G418	+G418
None	0.113 ± 0.01	18 ± 3	4 ± 1	233 ± 60	10 ± 3
SFFV$_p$M (F4-6)	1.45 ± 0.09	3134 ± 188	450 ± 63	3514 ± 220	403 ± 35
Neo^R SFFV$_p$ (8a+H)	1.68 ± 0.1	3404 ± 361	3149 ± 204	3611 ± 353	3424 ± 300

The assay for CFU-E was performed as described by Fagg and Ostertag. Data are the mean of three independent experiments. The concentration of G418 used was 1.5 mg/ml. Only the benzidine-positive cells were scored as CFU-E in the 48 h assay.

A



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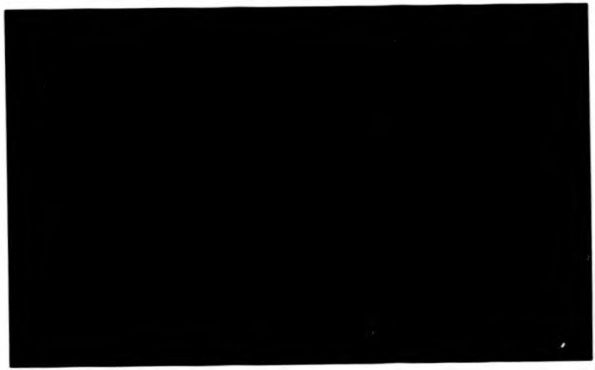
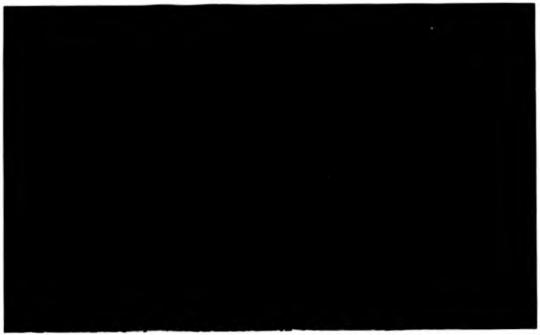




c

Figure 48. CFU-E induced by neo^R SFFVp are erythropoietin independent and G418 resistant.

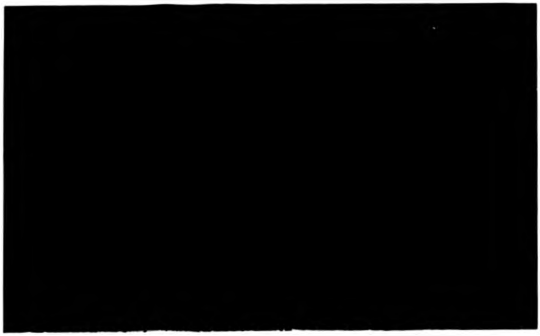
CFU-E colonies from the experiments depicted in Table 10 were photographed under 16x magnification, in cultures containing G418 and after benzidine staining.

A: Uninfected spleen CFU-E stained with benzidine in the presence of G418.

B: F4-6 infected spleen CFU-E stained with benzidine in the presence of G418.

C: neo^R SFFVp infected spleen CFU-E stained with benzidine in the presence of G418.

In these photographs the benzidine positive (B^+) colonies appear darker than the benzidine negative (B^-) cells although under the microscope the staining (i.e. blue for B^+) is more obvious.



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A: Uninfected spleen CFU-E stained with benzidine in the presence of G418.

B: F4-6 infected spleen CFU-E stained with benzidine in the presence of G418.

C: neo^R SFFVp infected spleen CFU-E stained with benzidine in the presence of G418.

In these photographs the benzidine positive (B^+) colonies appear darker than the benzidine negative (B^-) cells although under the microscope the staining (i.e. blue for B^+) is more obvious.

colonies, with up to several hundred cells. Almost all of the CFU-E synthesized haemoglobin in the presence of G418 and were thus B^+ in contrast to uninfected CFU-E or CFU-E infected with the prototype SFFV (Table 10; Figure 48). All CFU-E must have been infected with functional neo^R SFFVp conferring both erythropoietin independence and G418 resistance to the CFU-E cells.

e) Stability of neo^R SFFVp after serial in vivo passage

Co-expression of the neomycin resistance gene (neo^R) and the gp55 gene of SFFV, the latter of which is required for erythroid precursor cell transformation, was detected in all fibroblasts infected with neo^R SFFVp virus, even after viral passage. The data with CFU-E colonies in neo^R SFFVp infected mice, indicated that the majority, if not all, of the CFU-Es expressed coordinately growth factor independence and G418 resistance in contrast to previous SFFV vectors. To further confirm co-expression of the neo^R marker and the transforming gene (gp55) in virus infected transformed cells, mice were infected with neo^R SFFVp, and the virus was retested, after serial in vivo passage by the criteria outlined below.

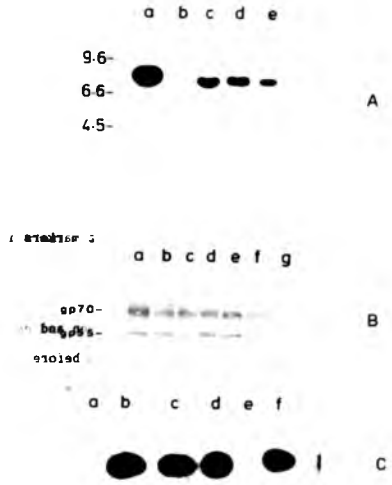
Mice infected with neo^R SFFVp were sacrificed eleven days p.i. and the spleens were removed and homogenised to give a single cell suspension. The cells were then pelleted and the resulting supernatant was titrated for virus activity both in vivo (spleen focus formation) and in vitro (GTU assay). From the cell fraction, DNA and protein was prepared for detailed analysis. The results of these experiments are shown in Table 11 and Figure 49.

Spleen passaged neo^R SFFVp always conferred G418 resistance to infected fibroblasts and induced the erythroid hyperplasia as expected.

Table 11. A stable increase in the SFFU/GTU ratio is found in in vivo passage of neo^R SFFV.

Passage	Spleen wt. (g)	GTU injected	GTU/spleen	SFFU/spleen	SFFU/GTU
Primary infection	1.2 ± 0.2	5.0×10^5	6.2×10^6	3.4×10^4	0.068
First passage	1.7 ± 0.22	6.2×10^5	1.2×10^7	8.5×10^4	0.14
Second passage	1.4 ± 0.12	1.7×10^6	1.2×10^7	1.9×10^5	0.11
Third passage	1.7 ± 0.3	1.3×10^6	2.0×10^7	1.7×10^5	0.13

DBA/2J mice were inoculated with 0.5 ml of virus dilution i.v.. Eleven days post infection mice were sacrificed, spleens were removed, and foci were counted. Spleens were then homogenized in a total of 5 ml and supernatants were titrated, both in vitro (GTU) and in vivo. (SFFU/GTU is the ratio of SFFU/spleen : GTU injected).


a b c d e
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4.5-
A
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B
a b c d e f
C

Figure 49. Serial passage of neo^R SFFVp in vivo

a) Southern blot analysis of spleen genomic DNA after serial passage of neo^R SFFV in DBA/2J mice. Genomic DNA (10 µg) was digested with SstI and then analysed by Southern blotting and hybridisation to a neo specific probe.

Lane a: neo^R SFFVp plasmid DNA (pMF16, 80 pg).
Lane b: DBA/2J spleen DNA.
Lane c: neo^R SFFVp passage 1 spleen.
Lane d: neo^R SFFVp passage 2 spleen.
Lane e: neo^R SFFVp passage 3 spleen.

The numbers to the left represent molecular weight markers in kb, lane M.

b) Western blot analysis of serially passaged spleen and control cell lines. Protein extracts were separated by SDS-PAGE before being transferred to Gene Screen Plus and probed with a goat anti-Friend virus antiserum.

Lane a: F4-6 infected spleen.
Lane b: F4-6.
Lane c: RAT1 neo^R SFFVp #8a+H.
Lane d: neo^R SFFVp passage 1 spleen.
Lane e: neo^R SFFVp passage 2 spleen.
Lane f: neo^R SFFVp passage 3 spleen.
Lane g: DBA/2J spleen.

c) Neomycin phosphotransferase assay.

Lane a: DBA/2J spleen.

Lane b: neo^{R} SFFVp spleen passage 1.

Lane c: neo^{R} SFFVp spleen passage 2.

Lane d: neo^{R} SFFVp spleen passage 3.

Lane e: F4-6 infected spleen.

Lane f: RAT1 neo^{R} SFFVp #8a-H.

For this analysis 100 µg aliquots of protein were assayed for functional neomycin phosphotransferase as described in Materials and Methods.

However, surprisingly an altered ratio of GTU transfer to fibroblasts and of spleen focus formation in the animal after one passage was seen in all experiments. The relative spleen focus forming capacity (SFFU/GTU) of the neo^R SFFVp was thus increased (Table 11).

The reasons for the biological alterations induced in neo^R SFFVp after passage in vivo are unclear, although no gross alterations at the DNA proviral genome level could be detected. Southern blot analysis of infected spleen cells showed predominantly an 8 kb band which hybridised to a neo specific probe after digestion with the restriction enzyme SstI (Figure 49a). No aberrant hybridising bands were detected, thus the majority if not all of the integrated neo^R SFFVp copies were of the correct size.

Levels of gp55 expression were comparable to those found in F4-6 virus infected spleens as shown by western blot analysis depicted in Figure 49b. Neomycin phosphotransferase enzyme analysis of infected spleens showed that the neo gene was also being transcribed and translated in vivo (Figure 49c). This analysis suggested that no segregation had occured between the neo and the env gene although it could not be ruled out that such infected spleens did indeed contain subsets of virus expressing only the neo gene or alternatively the env (gp55) gene.

To analyse this point in further detail, end point dilution of neo^R SFFVp virus was made on fibroblasts utilising G418 as a selectable marker for detecting neo^R SFFVp infected cells. G418 resistant fibroblast non-producer clones obtained from these end point dilutions of in vivo passaged virus were grown up and analysed for gp55 expression and integrity of the integrated proviral DNA genome. All of the 10 clones, which had one full sized copy of the neo^R SFFVp virus as

seen by Southern blot analysis also expressed gp55 (Figure 50).

Virus rescued from these clones and used to infect DBA/2J mice generated leukaemia and displayed the same altered SFFU/GTU ratio (Table 12).

These data reinforced the interpretation that co-expression of the neo^R and gp55 genes were stable functions of the neo^R SFFVp virus, although mutations leading to alterations of gene expression (presumably) occured and were selected during mouse passage to confer maximal transformation potential.

f) Neo^R SFFVp can be used to generate permanent Friend leukaemia cell lines which are resistant to G418

Although it is possible to establish transplantable cell lines from tissues infected with SFFV in the later stages of the disease, most of the proliferating cells early in the disease have a limited self renewal capacity and fail to grow in syngeneic donor mice or as cell lines *in vitro*. Tambourin and Wendling (1971) therefore proposed that the disease induced by SFFVp consists of multiple stages:
i) an early stage in which the accumulating erythroid precursor cells have a limited life span and the size of this proliferating compartment is controlled by removal of excessively proliferating cells,
ii) a late stage (more than three weeks) in which truly malignant cells proliferate and are capable of autonomous and tumourigenic growth *in vivo*.

The reasons for the emergence of the malignant cells are still obscure, even though a requirement for SFFV expression has been shown (Wolff *et al*., 1986; Ostertag *et al*., 1987).

It was thus decided to examine the potential of neo^R SFFVp in

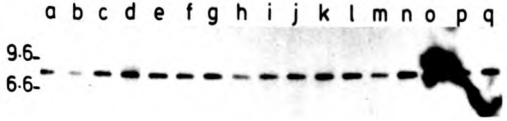

a b c d e f g h i j k l m n o p q
9·6
6·6

Figure 50. Southern blot analysis of G418 resistant RAT1 clones after end point dilution of in vivo passaged neo^R SFFVp

Genomic DNA (10 µg) from end point dilution RAT1 clones was digested with SstI prior to agarose gel electrophoresis and Southern blotting. The filter bound DNA was then hybridised with a ^{32}P radiolabelled neo specific probe.

The analysis of 16 (from a total of 30) clones is depicted below.

Lane a: to lane p: neo^R SFFVp infected RAT1 clones (p1 to p16).

Lane q: RAT1 neo^R SFFVp #8a+H.

Table 12. Biological properties of in vivo passaged neo^R SFFVp released by clones after end point dilution

Clone	neo^R SFFVp virus release			gp55
	GTU/ml	SFFU/ml	SFFU/GTU	expression
p1	1×10^8	9×10^3	0.09	+
p2	3×10^8	3.3×10^4	0.11	+
p3	1×10^8	1.2×10^4	0.12	+
p4	8×10^4	1.3×10^4	0.16	+
p5	7×10^8	5.6×10^4	0.08	+
p6	1×10^8	1×10^8	0.1	+
p7	8×10^8	7.2×10^4	0.09	+
p8	9×10^8	1×10^8	0.11	+
p9	3×10^8	3.9×10^4	0.13	+
p10	2×10^8	2.8×10^4	0.14	+

transforming erythroid precursors to malignancy in order to test questions related to malignancy and co-expression of SFFV function and differentiation.

DBA/2J mice were infected with 1 x 10^6 GTU neo^R SFFVp (from the cell line RAT1 neo^R SFFVp #8a+H) i.v.. After 3-4 weeks, surviving mice were sacrificed, the spleens were removed, and cellular homogenates were prepared. Cells were plated at a density of 5 x 10^5/ml in GMEM + 10% FCS, and were fed at 3 day intervals by removing part of the cell culture medium and replacing with new medium. After 2-4 weeks clones appeared and expanded at a fast rate. From six mice eight uncloned Friend cell lines (NF1 to NF8) were obtained. All of these cell lines, when cultured in medium supplemented with 2 mg/ml G418 (a concentration which is toxic for Friend cells, i.e. F4-6, B8/3), were resistant to the drug.

Further analysis of these Friend cells (Table 13) demonstrated that they all had a high cloning efficiency in agar, (in the presence of 2 mg/ml G418) a property of normal FLC.

Southern blot analysis of genomic DNA isolated from these cell lines, digested with SstI yielded fragments consistent with the presence of only full length virus in most cell lines, after hybridisation with neo (Figure 51). Western blot analysis also revealed that gp55 was being synthesized in large amounts in these cell lines.

Titration of tissue culture supernatants, from the cell lines NF1 to NF8, in the GTU assay showed that between 1 x 10^3 and 1 x 10^6 GTU/ml of neo^R SFFVp virus was being released (Table 13), whose biological activity was identical to that of the neo^R SFFVp virus passaged in mice, and differed in the SFFU/GTU ratio from the original vector virus (RAT1 neo^R SFFVp #8a+H, Table 11).

Table 13. Properties of geneticin resistant Friend cells transformed by neo^R SFFV$_p$.

Cell line	Cloning efficiency in agar + 2 mg/ml G418 (%)	Neo^R SFFV$_p$ Virus release GTU/ml	SFFU/ml	SFFU/GTU	gp55 expression	DMSO induction % benzidine positive cells[a]
NF-1	65	5 x 10^5	6.5 x 10^4	0.13	+	50
NF-2	50	2 x 10^5	3.2 x 10^4	0.16	+	60
NF-3	57	4 x 10^4	5.6 x 10^3	0.14	+	51
NF-4	70	1 x 10^4	n.d.[b]		+	43
NF-5	61	1 x 10^5	n.d.		+	62
NF-6	63	2 x 10^4	2.4 x 10^3	0.12	+	55
NF-7	60	1 x 10^5	1.1 x 10^4	0.11	+	65
NF-8	60	1 x 10^6	9 x 10^4	0.09	+	68

[a]Log phase Friend cells were induced to differentiate into hemoglobinizing cells by cultivation in 1.2% DMSO. After 5 days the percentage of hemoglobinizing cells was determined by staining with benzidine.

[b]Not done.


M a b c d e f g h i j k l M
23
9·6
6·6
4·5
2·3
2·0

Figure 51. Southern blot analysis of G418 resistant Friend cells

Genomic DNA (10 µg) from all Friend cell lines was digested with SstI before being analysed by agarose gel electrophoresis and Southern blotting. The filter was then hybridised with a neo specific ^{32}P labelled DNA probe.

Lane a: RAT1 neo^R SFFVp #8a+H.

Lane b: neo^R SFFVp plasmid DNA (pMF16, 80 pg).

Lane c: DBA/2J spleen.

Lane d: F4-6.

Lane e: NF-1.

Lane f: NF-2.

Lane g: NF-3.

Lane h: NF-4.

Lane i: NF-5.

Lane j: NF-6.

Lane k: NF-7.

Lane l: NF-8.

The numbers to the left represent molecular weight markers in kb, lane M.

All of these cells could also be stimulated (to varying degrees) to differentiate and synthesize haemoglobin in the presence of DMSO (1.5%, Table 13).

Discussion

The study of the specific cellular interaction of the SFFV coded glycoprotein requires experiments with defined erythroid target cells. These are only possible if selectable SFFV vectors can be utilised. Construction of selectable SFFV vectors have encountered unexpected difficulties utilising the SFFV genome alone (Joyner and Bernstein, 1983; Ostertag et al., 1987). Some of the constructs were unstable, some others showed lack of co-expression of the selectable marker gene and of gp55. In this study a selectable retrovirus vector, neo^R MPSV was used as a base to construct a hybrid viral vector with the SFFV env gene and the SFFV U3 region (neo^R SFFVp) of the LTR. This vector induced erythroleukaemia in animals, co-expressed the marker gene and the gp55 gene in fibroblasts and the marker gene and transforming functions in progeny of in vivo infected single erythroid precursor cells.

The only features of SFFV which are essential for retaining specific transformation potential, are the SFFV env region, including the env splice acceptor sequence of SFFV, together with the U3 region of the LTR. All other sequences within this virus originate from a MPSV vector, which has been shown to have no leukaemogenic effects both in vitro and in vivo, and is only able to transfer neo^R to fibroblasts (Stocking et al., 1985). This is an agreement with the data of others, who utilised an alternative approach. They retained as a base SFFV but

selected for *env* gene mutants, which could not induce erythroid transformation (Kabat *et al.*, 1980; Ruta *et al.*, 1983; Machida *et al.*, 1984, 1985a,b). These studies, however did not define SFFV sequences which may contribute to the high expression of gp55 in erythroid cells leading to transformation and the lower expression found in fibroblasts (Bilello *et al.*, 1980; Ostertag *et al.*, 1987). The elevated expression of gp55 in erythroid cells appears to be due to the large amounts of spliced *env* mRNA which is found in erythroid cells but not in fibroblasts (Bilello *et al.*, 1980). It may also be that the elevated expression of SFFV in erythroid cells is due to the U3 region (containing the enhancers), which may have an erythroid tissue specificity, as has been shown for F-MuLV (Bosze *et al.*, 1986). However, data (Wolff and Ruscetti, 1986) have shown that the pathogenicity of SFFVa is not dependent upon the LTR. However in these experiments, no expression studies *per se* were performed.

It may be interpreted that high expression and biological activity are determined by the *env* region, for the 3' region of the virus, including the *env* splice donor is derived from MPSV in this construct. Presence of the neo^R gene in the intron region which is also MPSV derived appears not to be responsible for high expression of gp55 in erythroid cells, although in the study of Joyner and Bernstein (1983b) deletion of sequences 3' to the *env* gene disrupted expression of gp55.

It would thus appear that either the *env* splice acceptor of SFFV or the gp55 gene itself is crucial for high erythroid expression of the SFFV genome. Replacement of the gp55 coding region and insertion of a reporter gene could distinguish between these alternatives.

The studies reported here, however, do not only contribute to the question which regions of SFFV are essential and which are contributory

to the specific inclusion of proliferation of erythroid precursor cells, they also open for the first time a way to define the target cell interactions required to elicit the SFFV specific erythroid response.

The biological activity of the neo^R SFFVp as compared to the wild type SFFVp is indistinguishable in every respect, including time course of the disease and organ involvement.

The dysfunction of CFU-E colonies (with respect to cell metabolism, i.e. haemoglobin production) in the short term CFU-E assay also potentiates the usefulness of this construct in defining the target cell for SFFVp.

This problem can now be approached utilising the selectable neo^R SFFVp described, in single haemopoietic precursor cell infections. For these experiments the helper free psi-2 and PA317 cell lines could be used. However, these cell lines do not produce very high titres (Table 8) although extended cell culture and selection could probably better the situation.

Although serial passage of neo^R SFFVp in adult DBA/2J mice results in no segregation of neo and gp55 expression or gross rearrangements of the viral genome, the fact that the SFFU/GTU ratio increases after one passage *in vivo* and that this ratio remained stable for the next two passages suggests that some form of selection had occured in the mouse, for a neo^R SFFVp that is able to confer G418 resistance and cause spleen foci more efficiently.

The second type of erythroid cell which is altered as a consequence of SFFVp infection is the transplantable tumour cell, which can also be passaged *in vitro*. These cells are arrested in differentiation in contrast to the undefined CFU-E precursor cells which differentiate

regardless of the presence of erythropoietin if infected by SFFVp *in vitro*.

Transformation of these cells requires the interaction of at least two mutations in the FLC. One is the requirement for SFFV expression and a second event which may be related to integration of SFFV or the helper virus close to sequences that result in a block in differentiation and/or immortalisation (Ostertag and Pragnell, 1982; Ostertag *et al*., 1987).

Reports by several groups have shown in a variety of Friend cell alterations in expression and structure of the p53 locus (Ruscetti and Scolnick, 1983; Shen *et al*., 1983; Rovinski *et al*., 1987). This could possibly be due to integration of SFFV near to one of the alleles, thus leading to dysfunction of p53, which is thought to be important in the control of the cell cycle (Rovinski *et al*., 1987). However, a second fraction of FLC are not altered in the expression of p53. The question then remains whether p53 activation is the primary event in causing malignancy or just a consequence of later mutations in the course of malignant progression. Recently, study of the integration sites of SFFV using an oligonucleotide probe (Moreau-Gachelin *et al*., 1986) has led to the finding that SFFVp integrates near to a putative oncogene Spi-1 in 95% of the tumours caused by this virus. Due to the prokaryotic nature of the neomycin gene, the neo^R SFFVp described provides a useful opportunity to analyse further the integration of SFFV in transformed FCLs which were obtained in this study.

Chapter 4

Analysis of neo^R SFFVp LTR functions

Introduction

The studies described in the previous chapter described the construction of a biologically active selectable F-SFFVp retroviral vector. Apparently from these studies only the env gene (gp55) in combination with the U3 region of F-SFFVp alone was required to cause pathological symptoms indistinguishable from the wild type F-SFFVp. Earlier studies which came to the same conclusion (Linemeyer et al., 1982) did however not exclude a role of the LTR in generating or reinforcing disease specificity. It has been shown that the U3 region of the MPSV LTR is crucial for expansion of the host range of this acutely oncogenic virus to some embryonal carcinoma cell lines (Hilberg et al., 1987) and for generating the MPSV specific myeloproliferative disease (Stocking et al., 1985, 1986). The MPSV U3 region moreover confers a higher transcription rate than the corresponding sequences of Mo-MuLV in dually permissive cells (W. Ostertag, pers. commun.).

Friend MuLV U3 sequences are required for the erythroid specificity of the Friend helper virus induced erythroproliferative disease (Chatis et al., 1984). The LTR of SFFV has retained features of both MCFV and F-MuLV (Ostertag et al., 1987). It was thus interesting to determine whether the U3 region of MPSV, which was utilised to generate a hybrid selectable neo^R SFFVp vector would alter the host range of SFFVp, or in view of the increased transcription rates accelerate the erythroid disease as compared to that elicited by the standard SFFVp. To this aim a new vector was constructed containing the U3 region from MPSV. This vector was designated neo^R SFFV-M. Similar studies, using the F-SFFVa strain (Wolff and Ruscetti, 1986) have shown that replacement of the

SFFVa LTR with that from Mo-MuLV did not alter the pathological outcome of the disease per se although no detailed analysis was carried out in this study.

Results

a) Construction of neo^R SFFV-M

A detailed description of how neo^R SFFV-M was constructed is shown in Figure 52. In this construct (pMFM#21) the 3' LTR, which was formerly of F-SFFVp origin (pMF16, neo^R SFFVp) was replaced by the 3' LTR of MPSV.

The Bal31 digestion step which was used to remove the F-SFFVp LTR sequences, was controlled by sequencing of the digested fragment after subcloning into a M13 vector (Figure 52). It can be seen from the sequence that all of the F-SFFVp LTR sequences had been removed without disturbing the gp55 coding sequence. The MPSV 3' LTR (subcloned as a HindIII fragment in pUC9, see Figure 53) from molecularly cloned MPSV has a partial p15E sequence 5' to it. However this sequence is separated from the stop codon in gp55 by a short sequence, corresponding to the polylinker cloning sequence from pUC19. From sequence data these sequences would not be translated even if the gp55 stop codon was overread. Thus neo^R SFFV-M is exactly the same as neo^R SFFVp except that the 3' LTR along with a small coding region 5' to it are of MPSV origin. In this vector the only sequences which are of F-SFFVp origin are the splice acceptor sequence and the gp55 coding sequence itself.

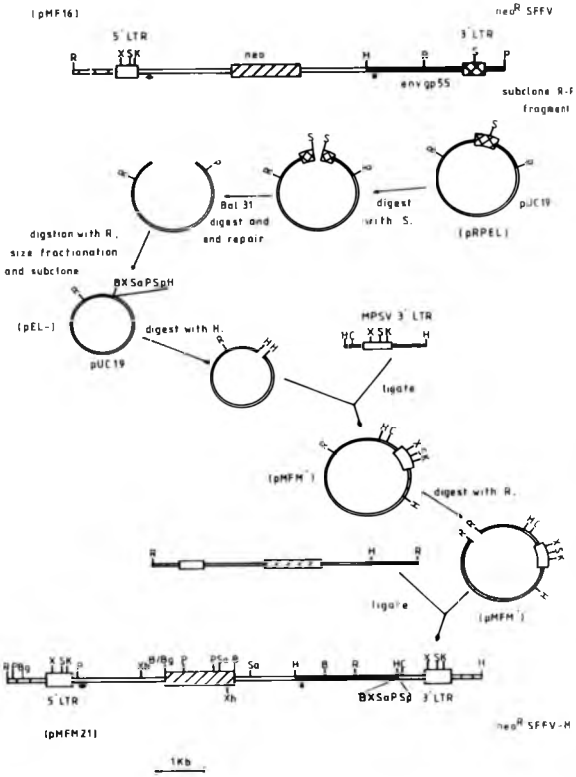

(pMF16)
neo^R SFFV
5' LTR
3' LTR
neo
env gp55
subclone R-F fragment
Bal 31 digest and end repair
digest with S.
pUC19
(pRPEL)
digstion with R, size fractionation and subclone
(pEL-)
pUC19
digest with H.
MPSV 3' LTR
ligate
(pMFM^-)
digest with R.
ligate
(pMFM^+)
5' LTR
3' LTR
(pMFM21)
neo^R SFFV-M
1Kb

Figure 52. Construction of the retroviral vector, neo^R SFFV-M

The retroviral vector neo^R SFFV-M (pMFM21), which differs from the original neo^R SFFVp vector, in that the 3' LTR is of MPSV origin, was constructed as follows.

Due to the absence of usable restriction enzyme sites within the non-coding region between the COOH terminus and the start of the SFFVp LTR, the LTR sequences were deleted by using the endonuclease Bal31. For this purpose, an EcoRI-PstI fragment encompassing the COOH terminus of env (gp55), the entire LTR and gag sequences of SFFVp origin, (from neo^R SFFVp, pMF16) was subcloned into pUC19 to give the plasmid pRPEL. This plasmid was then digested with SstI (which cuts within the LTR), and the resulting free ends were digested with Bal31, under the appropriate conditions. After preparation of blunt ends with Klenow, and EcoRI digestion of the Bal31 treated plasmid, fragments of the correct size (according to sequence data appr. 500 - 600 bp) were collected after electrophoretic separation.

These fragments were then cloned into EcoRI.SmaI digested pUC19. The resulting recombinants were then screened, after digestion with EcoRV and BamHI for inserts of appr. 220 bp. One such clone pEL-, had an EcoRV.BamHI insert of appr. 220 bp and was used for further analysis. The EcoRV.BamHI insert was subcloned into M13mp18, and subsequently sequenced as outlined in Materials and Methods.

This sequence analysis revealed that all of the LTR sequences had been removed, without disturbing the env coding region (see Figure 53). pEL- was then digested with HindIII and a HindIII fragment, encompassing a portion of the p15E sequence, the MPSV 3' LTR as well as genomic

flanking sequences (3' to the LTR), was ligated into pEL- to give rise to a new recombinant plasmid pMFM'.

pMFM' was digested with EcoRI and an EcoRI fragment from pMF16, delineating the 5' LTR (from MPSV), neo gene and the 5' env sequences was ligated into pMFM' to give rise to pMFM21 (neo^R SFFV-M).

The restriction enzyme single lettering code is

B, BamHI; Bg, BglII; C, ClaI; H, HindIII; K, KpnI; P, PstI; R, EcoRI; S, SstI; Sa, SalI; Sp, SpMI; X, XbaI; Xh, XhoI.

◆ Splide donor.

● Splice acceptor.


env
LTR
1)
env
ATAAGAATTAGTTAATCAAGTTTAAACAATTTCTGTCCTGGAGTCATCGGGTCCGAAATCAAGGACTGAGTTGTTATGGTGGTCGA
TATTCTTAATCAATTAGTTCAAATTTGTTAAAGACAGGACCTCAGTAGCCCAGGCTTTAGTTCCTGACTCAACAATACCACCAGCT
TyrSer
2)
pEL-
GTCATCAGGTCCGAAATCAGGACTGAGTTGTTATGGTG/CCCCTAG
CAGTAGTCCAGGCTTTAGTCCTGACTCAACAATACCAC/GGGGATC
3)
M13mp18
TAATGCTTAAGCTCGAGCCATGGGCCCCTAGGAGATCTCAGCTGGACGTCCGTACGTTCGAACCGTGACCGG
ATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCC
EcoRI SstI KpnI SmaI BamHI XbaI SalI PstI SphI HindIII HaeIII

Figure 53. Nucleotide sequence analysis of pEL-

Shown are the nucleotide sequences of:

1) *env*: This sequence represents the COOH terminus (the last two amino acids) of the *env* gene used for the construction of pEL-.
3' to the COOH terminus is the untranslated sequence which is 5' to the 3' LTR.

2) pEL-: This sequence represents the Bal31 digestion analysis and thus shows that all of the LTR has been removed without altering the *env* (gp55) gene sequence.

3) M13mp18: This sequence demonstrates the transition from the M13 polylinker (BamHI) to the cloned pEL- sequence.

Homologous sequences are joined by *

b) Construction of neo^R SFFV-M non-producer and producer cell lines

i) Southern blot analysis

To further test the biological activity of this new construct, non-producer and helper independent producer cell lines were constructed in a similar manner as described for neo^R SFFVp. Transient harvests (48 h) of supernatants from transfected psi-2 and PA317 cells were used to infect either psi-2 cells or F-MuLV producing NIH3T3 cells (3T3+M). Clones were selected for three weeks in GMEM + 10% FCS supplemented with 400 µg/ml G418. Southern blot analysis of SatI digested genomic DNA from individually picked clones (both psi-2 and 3T3+M) revealed that about 50% of the neo^R SFFV-M infected clones contained stably integrated viruses of the correct size (Figure 54). Further analysis of these clones revealed that virus titres (expressed as GTU/ml) of up to 10^6 GTU/ml were being released (Table 14).

ii) Coexpression of neo^R SFFV-M functions in fibroblasts

The cell lines neo^R SFFV-M 3T3+M#1, neo^R SFFV-M 3T3+M#7 and neo^R SFFV-M 3T3+M#9 (cells of which release a complex of both F-MuLV and SFFV-M) which released titres of 10^5- 10^6 GTU/ml, were then further analysed by western blotting to determine whether gp55 was being produced in these cells. The result depicted in Figure 55 indeed shows that neo^R SFFV-M infected clones synthesize gp55 in comparable amounts to that of neo^R SFFVp, although the amounts in neo^R SFFV-M 3T3+M#1 were barely detectable. The neo^R SFFV-M construct is, like the related neo^R SFFVp construct, able to confer G418 resistance to infected cells and synthesizes detectable amounts of gp55. That these cell lines contained a stable single integrated copy of neo^R SFFV-M, again shows that there has been no segregation between neo^R and gp55 expression.

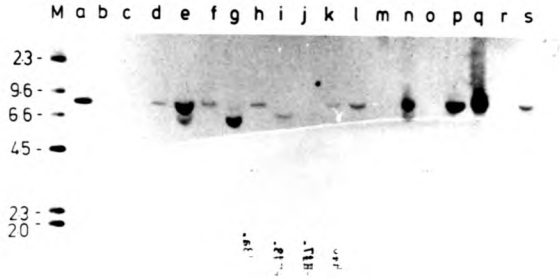

M a b c d e f g h i j k l m n o p q r s
23-
96-
66-
45-
23-
20-

Figure 54. Southern blot analysis of neo^R SFFV-M infected NIH 3T3+H and psi-2 clones

Genomic DNA (10 µg) from the individual clones was digested with SstI before being analysed by agarose gel electrophoresis and Southern blotting. The filter was hybridised with a ^{32}P labelled neo fragment.

Lane a: pMFM #21 (80 pg).

Lane b: psi-2.

Lane c: NIH 3T3+H.

Lane d: neo^R SFFV-M 3T3+H#1.

Lane e: neo^R SFFV-M 3T3+H#3.

Lane f: neo^R SFFV-M 3T3+H#5.

Lane g: neo^R SFFV-M 3T3+H#6.

Lane h: neo^R SFFV-M 3T3+H#7.

Lane i: neo^R SFFV-M 3T3+H#8.

Lane j: neo^R SFFV-M 3T3+H#8a.

Lane k: neo^R SFFV-M 3T3+H#9.

Lane l: neo^R SFFV-M psi-2#1.

Lane m: neo^R SFFV-M psi-2#2.

Lane n: neo^R SFFV-M psi-2#3.

Lane o: neo^R SFFV-M psi-2#4.

Lane p: neo^R SFFV-M psi-2#7.

Lane q: neo^R SFFV-M psi-2#8.

Lane r: neo^R SFFV-M psi-2#5.

Lane s: neo^R SFFV-M psi-2#9.

The numbers to the left represent molecular weight markers in kb, lane M.

Table 14. GTU titres being released from neo^R SFFV-M infected cells

Cell Line	GTU/ml*
neo^R SFFV-M 3T3+H#1	1 x 10^5
neo^R SFFV-M 3T3+H#5	5 x 10^5
neo^R SFFV-M 3T3+H#7	1 x 10^6
neo^R SFFV-M 3T3+H#9	7 x 10^5
neo^R SFFV-M psi-2#1	1 x 10^5
neo^R SFFV-M psi-2#7	1 x 10^4
neo^R SFFV-M psi-2#8	3 x 10^3
neo^R SFFV-M psi-2#9	1 x 10^4

* All supernatants titrated on RAT1 cells as described in Materials and Methods. Titres represent the mean of triplicate dilutions.


a b c d e f g
gp70
gp55

Figure 55. Western blot analysis of neo^R SFFV-M infected fibroblasts

100 µg samples of protein lysates prepared from the various clones were analysed by SDS PAGE in a 10-30% gel system as described in Materials and Methods. The separated protein were then electro-transferred to a nitrocellulose filter. The filter bound proteins were then hybridised with an anti-Friend virus goat anti-serum. Specific bands were visualised by incubating with a peroxidase conjugated anti-goat antiserum.

Lane a: RAT1

Lane b: F4-6

Lane c: neo^R SFFV-M 3T3+H #1

Lane d: RAT1 pcl-5 1L#6

Lane e: neo^R SFFV-M 3T3+H #7

Lane f: neo^R SFFV-M 3T3+H #9

Lane g: RAT1 neo^R SFFVp #8a+H

The virus released from these cell lines was also able to confer G418 resistance, and underwent no detectable genetic alterations after transfer to RAT1 cells as seen by Southern blot analysis, as described for neo^R SFFVp (data not shown).

c) Neo^R SFFV-M causes an acute erythroid hyperplasia in susceptible mice, similar to neo^R SFFVp, but with a different kinetics

From the data presented above it appeared that like the neo^R SFFVp vector neo^R SFFV-M was capable of dual expression of G418 resistance and gp55 synthesis, after stable transfer in fibroblast cells. It was thus interesting to see whether the LTR change from SFFVp to MPSV U3 specific control sequences had any diverse effect upon the biology/pathology of this acutely transforming virus. To this end the following in vivo experiments were performed.

Serial dilutions of virus being released from the cell lines F4-6 (which served as an internal positive control) RAT1 neo^R SFFVp #8a-H and neo^R SFFV-M 3T3#9, were inoculated i.v. into groups of six female DBA/2J mice. Eleven days post infection the mice were sacrificed, spleens were removed, weighed, fixed in alcohol and the foci were counted. The results from this preliminary experiment indicated that both neo^R SFFVp (as shown previously) as well as neo^R SFFV-M induced splenomegaly (although to varying extents) and spleen foci (SFFV) if the same titre of biologically active virus particles as determined by fibroblast GTU (Table 15) was injected. The ratio GTU/SFFU was similar for both constructs if mice were sacrificed at 11-16 days post infection. However, in a parallel set of experiments, in which the same virus inoculums were used, but in which the mice were sacrificed at day 8-11, the spleen foci, induced by neo^R SFFV-M were difficult to score,

Table 15. Neo^R SFFV-M is not as efficient as neo^R SFFV$_p$ or SFFV$_H$ in inducing splenomegaly and spleen foci

GTU injected	Neo^R SFFVp (8a+H)			Neo^R SFFV-M #6			Neo^R SFFV-M #9		
	Spleen wt.^{a} (g)	SFFU	SFFU/GTU	Spleen wt.^{b} (g)	SFFU	SFFU/GTU	Spleen wt.^{b} (g)	SFFU	SFFU/GTU
5 x 10^5	1.2 ± 0.2	n.d.	-	0.6 ± 0.1	n.d.	-	0.5 ± 0.11	n.d.	-
1 x 10^5	0.8 ± 0.1	n.d.	-	0.24 ± 0.07	n.d.	-	0.3 ± 0.09	n.d.	-
2 x 10^4	0.53 ± 0.11	n.d.	-	0.16 ± 0.03	n.d.	-	0.2 ± 0.0	n.d.	-
4 x 10^3	0.25 ± 0.09	n.d.	-	0.15 ± 0.01	n.d.	-	0.15 ± 0.04	n.d.	-
8 x 10^2	0.13 ± 0.03	56 (42.59.63.59)	0.07	0.1 ± 0.02	42 (45.51.40.33)	0.05	0.09 ± 0.03	51 (42.57.60.44)	0.06

Data shown represent the mean of four values.

^{a}Mice were sacrificed at day 11 for neo^R SFFV$_p$ or ^{b}at day 16 for neo^R SFFV-M infected mice: Spleen foci could not be quantitated in these mice at day 11.

n.d.; not done, foci were too numerous to count.

The numbers in brackets reperesent actual spleen foci counted at that dilution.

and also the spleen weights were significantly lower as compared to neo^R SFFVp (or even F4-6), when equivalent virus titres (GTU) were inoculated.

To distinguish whether neo^R SFFV-M caused a qualitatively identical or even a different disease as compared to wild type SFFVp and neo^R SFFVp, a separate series of mice were infected with the two viral constructs as well as the standard SFFVp (F4-6).

At different times post injection (day 4, 9 and 16) mice were sacrificed and the spleens were removed for histological study as well as for in vitro colony assays. The results of this analysis are shown in Table 16 and Figure 56. The erythroproliferative disease, typical of SFFVp was observed in all of the animals infected with SFFVp (F4-6, neo^R SFFVp and neo^R SFFV-M). However, the erythroproliferative disease caused by neo^R SFFV-M (expansion of epo independent and G418 resistance CFU-E), was somewhat delayed in the spleens of these infected animals, although an equivalent titre was inoculated as compared to neo^R SFFVp.

Histological examination of the spleen, showed that all three viruses caused an erythroproliferation in the red pulp at early phases post infection. At day 16, SFFVp and neo^R SFFVp in contrast to neo^R SFFV-M infected spleens showed already signs of necrosis and a large proportion of immature erythroblasts with very little active erythropoiesis. Spleens infected by neo^R SFFV-M thus had retained a similar histological picture at a later phase as compared to the pathological picture seen in an earlier phase of the disease, for SFFVp and neo^R SFFVp thus suggesting also at this level that although the same viral titres were being inoculated the disease kinetics were indeed notably different. One very interesting observation seen in the white pulp of neo^R SFFVp and neo^R SFFV-M but not SFFVp (F4-6) was the

Table 16. Neo^R SFFV$_p$ vectors induce an increase in erythropoietin independent erythroid precursor cells.

Days p.i.	Virus[a]	Spleen wt.	Benzidine-positive CFU-E/10^5 spleen cells			
			(-)-Epo[b]		(+)-Epo	
			-G418	+G418	-G418	+G418
4	None	0.11 ± 0.03	14 ± 5	6 ± 3	277 ± 27	22 ± 7
	SFFV$_p$	0.4 ± 0.15	1403 ± 237	127 ± 13	1639 ± 322	151 ± 27
	Neo^R SFFV$_p$	0.42 ± 0.13	1833 ± 331	1597 ± 132	2011 ± 331	1956 ± 209
	Neo^R SFFV-M	0.15 ± 0.04	493 ± 87	376 ± 57	515 ± 98	485 ± 111
9	None	0.08 ± 0.02	18 ± 4	4 ± 2	317 ± 42	52 ± 12
	SFFV$_p$	0.7 ± 0.21	5144 ± 315	513 ± 37	5803 ± 436	524 ± 27
	Neo^R SFFV$_p$	0.8 ± 0.23	6144 ± 478	5981 ± 417	6873 ± 487	6153 ± 510
	Neo^R SFFV-M	0.32 ± 0.13	1578 ± 411	1103 ± 153	1829 ± 218	1543 ± 216
16	None	0.11 ± 0.03	22 ± 8	8 ± 3	514 ± 53	84 ± 12
	SFFV$_p$	1.4 ± 0.35	12144 ± 531	819 ± 176	11803 ± 417	827 ± 101
	Neo^R SFFV$_p$	1.6 ± 0.37	14163 ± 476	11824 ± 531	16791 ± 516	14276 ± 546
	Neo^R SFFV-M	0.77 ± 0.21	7872 ± 221	7974 ± 451	8448 ± 433	6532 ± 302

[a]All mice were infected with either 1 x 10^5 GTU (neo^R $SFFV_p$, neo^R SFFV-M) or, in the case of the wild type $SFFV_p$, 1 x 10^4 SFFU.

[b]Erythropoietin.

A

B

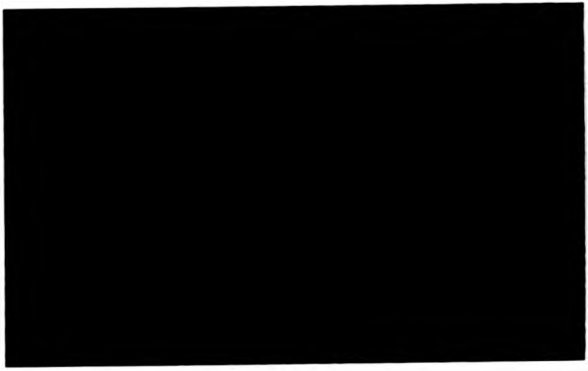
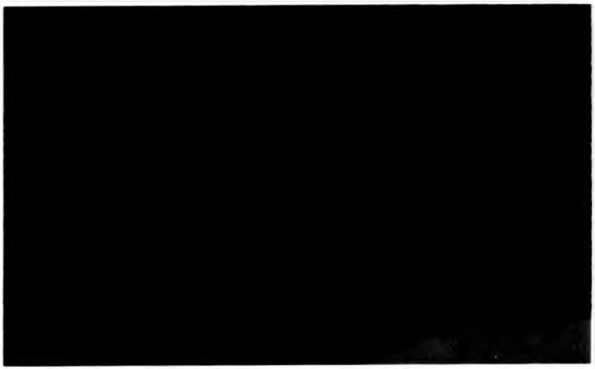


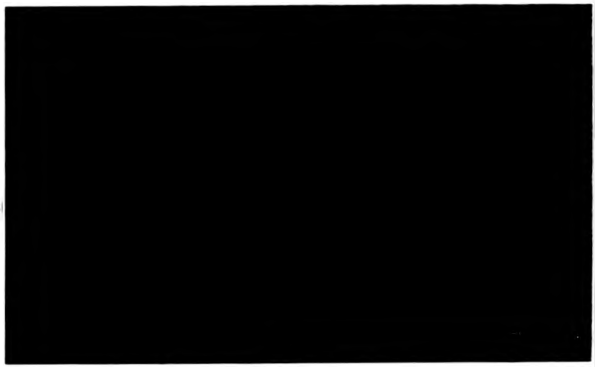
Figure 56. **The kinetics of the histopathological effects of neo^R SFFVp and neo^R SFFV-M in the spleen of DBA/2J mice**

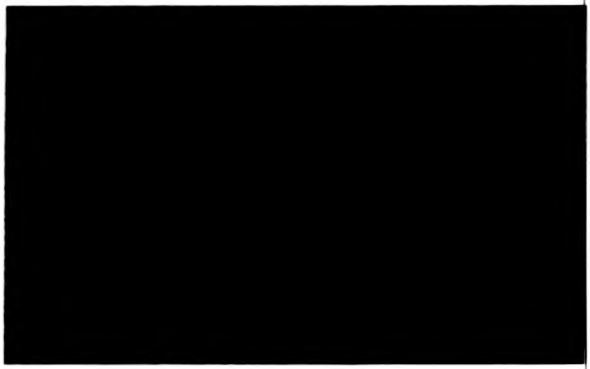
56.1. Section of an uninfected DBA/2J mouse spleen demonstrating the cell content of both the white (WP) and the red pulp (RP) (A, photographed at 25x magnification) and a more detailed look at the subcapsular region of the red pulp (B, 40x magnification). Active erythropoiesis can be seen (E) in the red pulp as well as various differentiation stages in erythropoiesis. M represents normocytes and MZ is the marginal zone. Sections were stained with Giemsa.

A



B





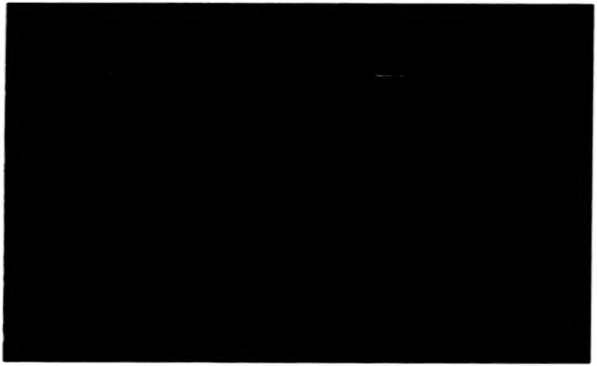
56.2. Day four[illegible]t infection. (all 25x after staining with haemolysin and eosin)

A. SFFVp. A section of the red pulp in which a cluster of erythroblasts (Eb) can be seen just below the connective tissue (CT) of the spleen. Again areas of active erythropoiesis can be seen (E).

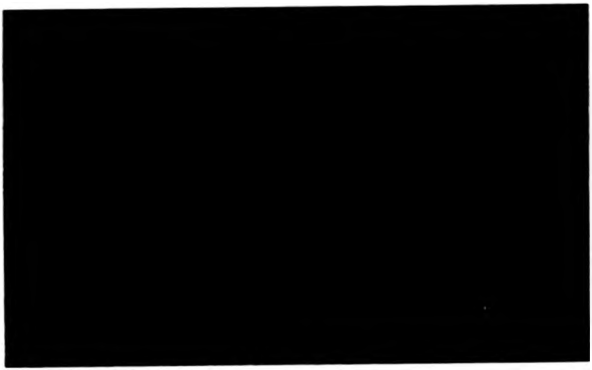
B. neo^R SFFVp. Section [illegible] red pulp to demonstrate two foci of erythroblast like cells, one focus of which is in the subcapsular space of the red pulp. Compared to SFFVp the foci are somewhat larger and more numerous.

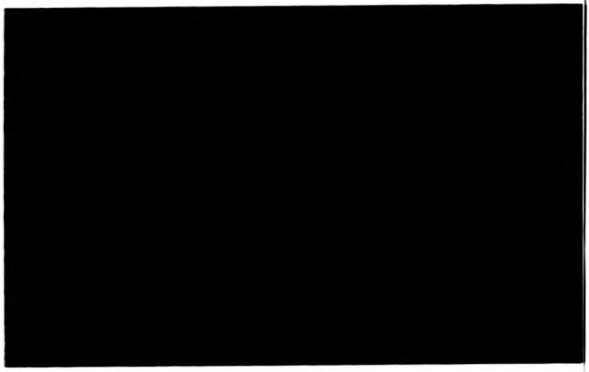
C. neo^R SFFV-M. Foci of erythroblasts are very hard to find and are not numerous in the red pulp.

A



B





C

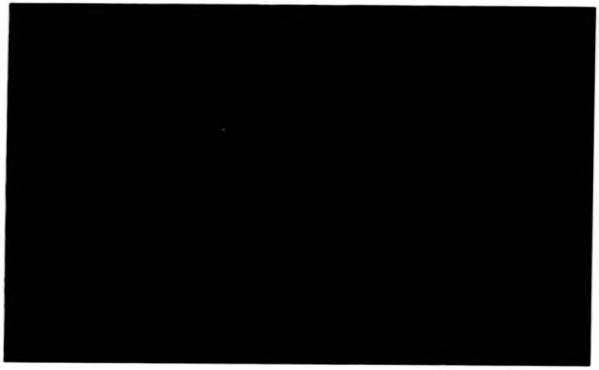
56.1. Day nine post infection (40x, HE)

A. SFFVp. The red pulp is full of erythroblasts, the foci being confluent. Islands of normal erythropoiesis (E, erythrocytes and normocytes) can still be seen.

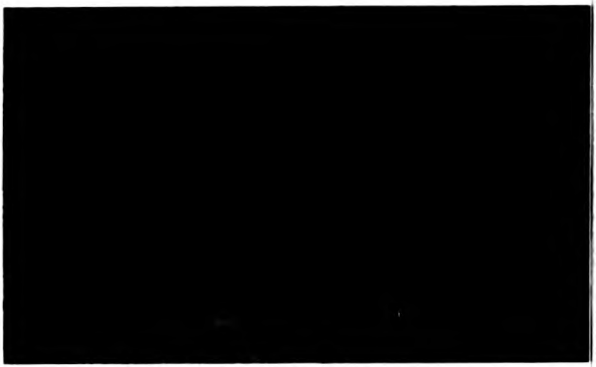
B. neo^{R} SFFVp. This section of the red pulp demonstrates that the predominant cell types are erythroblasts, with very few normocytes and mature erythrocytes, thus demonstrating radical proliferation of immature erythroid cells. There is less differentiation as compared to the SFFVp and neo^{R} SFFV-M infected mice.

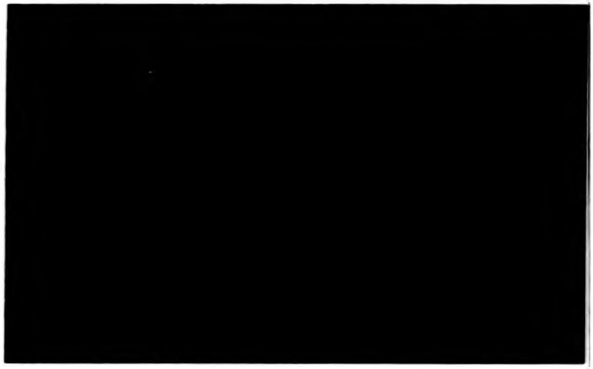
C. neo^{R} SFFV-M. The picture seen here represents an intermediate of the above sections. Areas of erythropoiesis can be seen. There is overall more differentiation to be seen. Numerous erythroblasts can also be seen.

A



B





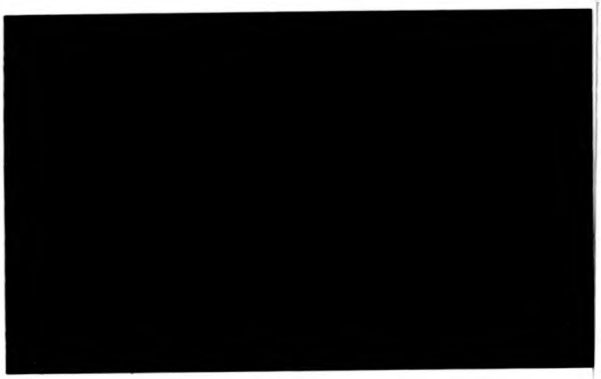
56.4. Day sixteen post infection (25x, HE)

A. SFFVp. Numerous blasts can be seen although there is more normal erythropoiesis (more differentiation) as compared to neo^s SFFVp.

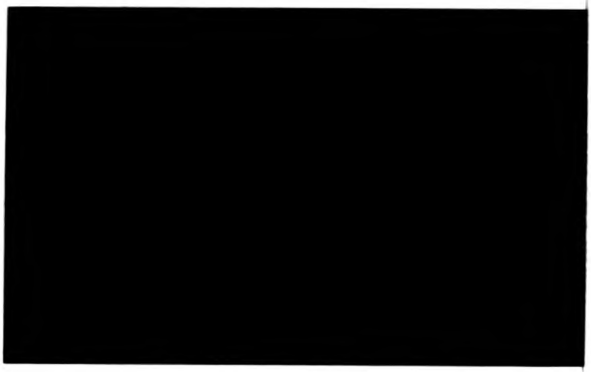
B. neo^s SFFVp. Larger blasts with fewer normocytes and less differentiated erythropoiesis.

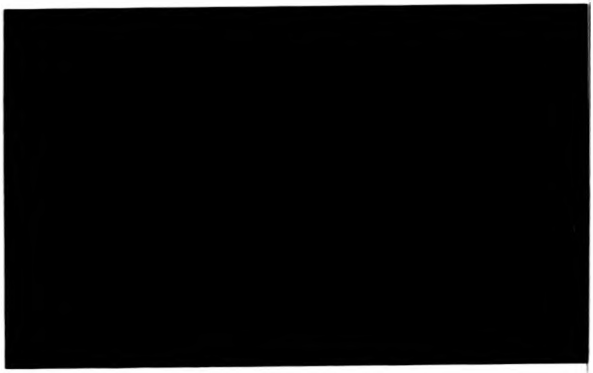
C. neo^s SFFV-M. The picture seen here resembles that seen for neo^s SFFVp at day nine.

A



B





56.5. Histopathology in the white pulp of neo^{R} SFFVp infected spleens (day 16)

A. This section (40x, Giemsa) demonstrates the presence of erythroblasts and areas of active erythropoiesis in the white pulp. Various granulocytes (G), histiocytes (H) and monocytes can also be seen.
The picture seen demonstrates a general stimulation of myelopoiesis.

B. This section of the white pulp (16x, acid phosphatase) demonstrates the extensive proliferation of histiocytes.

C. A section of a normal uninfected spleen (16x) after alkaline phosphatase staining demonstrating the distribution of mononuclear monocytes in the WP and RP.

infiltration of erythroid blasts and to some extent mature erythroid cells. This effect was more pronounced in day 16 neo^R SFFVp infected spleens than in neo^R SFFV-M infected animals (Figure 56.5, A and B). A parallel observation which also seemed to be specific for the neo^R SFFVp vectors (also neo^R SFFV-M) was a dramatic proliferation of histiocytes in the white pulp which was not seen for SFFVp (Figure 56.6).

d) Construction of a neo^R SFFVp based retroviral vector

From the data presented in this and the previous chapter it can be seen that the neo^R SFFVp and the neo^R SFFV-M constructs are both biologically active and stable after both *in vitro* and *in vivo* infections. It has also been seen that the *env* gene in these vectors (notably neo^R SFFVp, gp55) is expressed at high levels both *in vitro* and *in vivo* in cells of the erythroid pathway (i.e. CFU-E and Friend cells). From the constructs presented here, it appears that the high level of expression of the gp55 gene, is either a consequence of more efficient splicing of this subgenomic mRNA, or is indeed a property of the *env* gene *per se*. Thus to further analyse this question it was decided to construct a neo^R SFFVp based vector in which the gp55 *env* gene is deleted and replaced by restriction enzyme recognition sites which could be used for the insertion of respective genes. The construction of this vector is shown in Figure 57. This construct differs somewhat from the original neo^R SFFVp vector in that the neo^R MPSV coding sequences, including part of the *neo* gene have been deleted. This was undertaken, to: 1) Remove restriction enzyme sites which were be used for the cloning of genes into the vector (notably *EcoRI* and *BamHI*). 2) To reduce the size of the vector so that larger

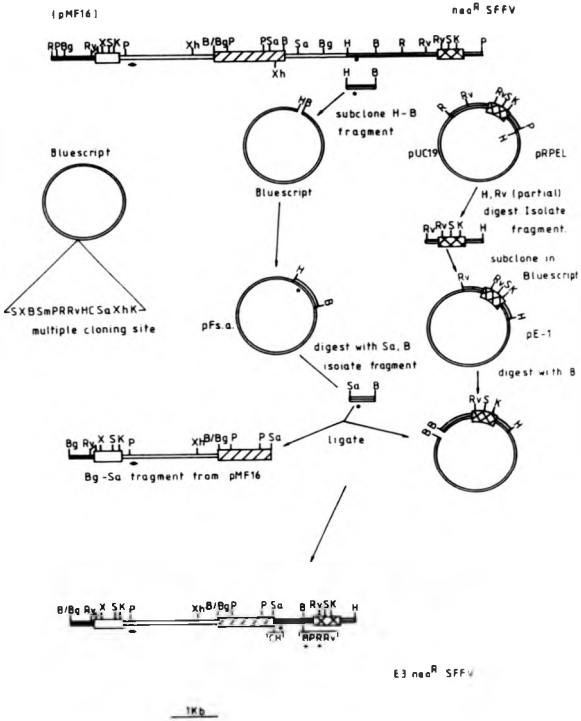

(pMF16)
neo^R SFFV
Xh
H B
subclone H-B fragment
Bluescript
pUC19
pRPEL
H, Rv (partial) digest Isolate fragment.
subclone in Bluescript
multiple cloning site
pFs.a.
digest with Sa, B
isolate fragment
pE-1
digest with B
Sa B
ligate
Bg-Sa fragment from pMF16
E3 neo^R SFFV
1Kb

Figure 57. Construction of a neo^R SFFVp based retroviral vector, E3

The E3 vector was constructed as follows:

1) pRPEL (from the construction of neo^R SFFV-M) was digested with HindIII and then partially digested with EcoRV. The resulting fragment (COOH terminus of gp55, and the 3' LTR) was cloned into "Bluescript" (pE-1).

2) The HindIII.BamHI 600 bp fragment which contained the splice acceptor sequence and none of the env coding sequence, was cloned into the same sites in the "Bluescript" cloning vector (pFs.a.).

The splice acceptor sequence was then isolated after digestion with BamHI and SalI (see polylinker site of Bluescript) and ligated together with BamHI digested (and "cipped") pE-1 and a BglII.SalI fragment isolated from pMF16.

The single letter restriction enzyme code is as follows:
B, BamHI; Bg, BglII; C, ClaI; H, HindIII; K, KpnI; P, PstI; R, EcoRI; Rv, EcoRV; S, SstI; Sa, SalI; Sm, SmaI; X, XbaI; Xh, XhoI.

◆ Splice donor.

● Splice acceptor.

- represent cloning sites in the E3 vector.

sequences could be introduced into the vector, without leading to problems when packaging the subsequent viral RNA into virions. The scheme used involved several subcloning experiments in the bacterial cloning vector "Bluescript", whose polylinker sequence enabled the introduction of the EcoRI site into the E3 vector. Further subcloning of the splice acceptor region of neo^R SFFVp, encompassed within the HindIII BamHI fragment, into "Bluescript" enabled the excision of this sequence with SalI and BamHI terminal sequences, which were compatible to the other ends of the fragments used in this construction.

This vector thus offers a unique opportunity to examine whether the high expression of the gp55 env protein in erythroid cells is a property of the protein per se or whether the elevated splicing of this subgenomic message is a property of the SFFVp splice acceptor sequences. Insertion of indicator genes within the polylinker cloning site may well aid to analyse this question.

Discussion

It has been demonstrated in the previous chapter that a retroviral vector containing a dominant selectable marker gene, the env gp55 gene (including its splice acceptor sequence) and the SFFVp U3 region is able to cause a disease indistinguishable from the wild type SFFVp. This virus neo^R SFFVp was also able to confer G418 resistance to infected cells. From this study it could be concluded that only the env gene (gp55) from SFFVp along with the U3 are needed to give an erythroproliferative disease, for the rest of the retroviral genome, was derived from neo^R MPSV which has been shown to cause no detectable pathological symptoms in vivo as well as in vitro. It was thus

interesting to ask whether the U3 region is responsible for the tissue specificity/pathogenicity of the retrovirus as has been shown in other systems (Chatis et al., 1984; Stocking et al., 1985, 1986).

A similar study performed by Wolff and Ruscetti (1986), using a SFFVa construct, demonstrated that the LTR did not determine the disease specificity of SFFVa per se. The results presented here substantiate this finding, in that neo^R SFFV-M was able to cause an erythroproliferative disease, whose target cells (CFU-E) were also erythropoietin independent and G418 resistant. However in all of the experiments performed where both neo^R SFFVp and neo^R SFFV-M were inoculated in parallel and in equivalent titres, there was always a "lag" in the disease caused by neo^R SFFV-M, as determined by spleen weight, colony assays or at the histopathological level. Although the neo^R SFFV-M vector was apparently less efficient than the neo^R SFFVp construct it was also able to stimulate the infiltration of immature erythroid cells into the white pulp of the spleen and also cause a massive proliferation of macrophages again in the white pulp. This was not seen for the wild type SFFVp and may thus reflect that part of the genome which is not of SFFVp origin is able to cause this effect although further explanations for this are at the moment possible.

Analysis of the stability of neo^R SFFV-M in vitro showed that the construct, was stably integrated in the cell lines used in this study, and that the virus was stably transferred to RAT1 fibroblasts. This analysis (Southern blotting) was unable to detect any changes in the genome structure of neo^R SFFV-M. Thus it may be concluded that the lag in the disease, was caused by the LTR change. A possible reason for this may be a decreased transcription rate within the target cells (i.e. those of the early erythroid pathway), although MPSV itself is

able to cause an increase of CFU-E (and also BFU-E) within the spleen, thus demonstrating that the LTR is functional within these cells. However, the MPSV disease, is caused by the *mos* oncogene (in conjunction with the U3 region, Stocking *et al*., 1985, 1986), which apparently needs only be expressed at very low levels in order to cause transformation either *in vitro* or *in vivo*. High expression of *mos* appears to be toxic for the cell. These observations may thus help to explain the phenomenon seen with the neo^R SFFV-M vector. It may well be that the SFFVp in order to cause its rapid erythroproliferative disease is dependent upon high levels of gp55 expression within the infected target cells. This reduced expression, within the early erythroid compartment from the MPSV LTR may well lead to a lag in the disease kinetics, in that more time is needed to, a) reach the level of gp55 needed to cause the erythroproliferative, or b) be expressed in enough target cells. The basis for this expression is not obvious. The MPSV LTR sequence is very similar to that of Mo-MuLV (Stacey *et al*., 1984; Ostertag *et al*., 1987; M. Grez, pers. commun.), which was used in the study of Wolff and Ruscetti (1986). However, one obvious difference, apart from several point mutations, between the MPSV and the SFFVp LTR is a deletion of one of the direct repeats from the U3 region of SFFVp. This deleted "enhancer" containing region, could thus possibly be responsible for the elevated expression of gp55 in the spleen of infected animals. A variant of MPSV, PCMV has also undergone deletion of one of the direct repeats, has an extended host range due to this deletion (as well as a further deletion in the *gag* region). Thus although the neo^R SFFV-M is able to cause the same erythroproliferative disease as neo^R SFFVp there is a difference in the kinetics, which may be assigned to the LTR. One interesting observation, however, is that

no transformed Friend cell lines could be isolated using this virus, even after repeated inoculation of susceptible mice with high virus titres and prolonged incubation periods (up to six weeks). The reason for this is unclear, for parallel experiments using neo^R SFFVp, consistently gave rise to transformed cells, after culture in vitro. Possible explanations could be that either not enough target cells were expanded, which could thus lead to the rare event of transformation, or that the proposed specific integration even of SFFVp (adjacent to the putative oncogene Spi) is LTR sequence specific, a consideration that is unlikely. It may well be a combination of both high expression of gp55 which leads to an erythroproliferative expansion and then subsequent selection for a transformed phenotype (i.e. Friend cells). In this respect the high levels of gp55 may well be critical, and a determining step for the expansion of the erythroid target cells. Although neo^R SFFV-M is able to cause the erythroproliferation, (although be it with a different kinetics), it may well be insufficient to allow for a selection and thus subsequent proliferation of the transformed cells. Indeed in some of the infected mice, there was a regression in the spleen weight after prolonged incubation periods with neo^R SFFV-M due to the host immune system. In this case it appears that the immune system is able to control the tumourogenic action of the neo^R SFFV-M virus.

From the data presented in this and also the previous chapter it appeared that the neo^R SFFVp, could well be an interesting vector for analysing gene expression within the erythroid pathway, although it is not clear whether the high expression of gp55 within erythroid cells is property of the gene itself, or of the splice acceptor sequences, together with the U3 of SFFVp. Thus to attempt to elucidate both of

these questions a neo^{R} SFFVp E3 retroviral vector was constructed, in which genes may be inserted behind the splice acceptor site in place of the gp55 gene (which has been in most part deleted).

This vector is being employed to look at the effects of epo, the SFFVa *env* gene, SFFVp *env* gene (neo^{R} SFFVp) and the R-SFFV *env* gene in stem cells, which are able to differentiate along the myeloid pathway (including the erythroid, N. Ahlers and A. Nowock, pers. commun.). Such studies may further define the biological effects of gp55, with respect to the erythropoietin independence/dependence of the target cell infected by these viruses.

General discussion

General discussion

Although F-SFFVp was one of the first highly oncogenic viruses to be isolated and indeed molecularly cloned, (Friend, 1957; Linemeyer et al., 1980) little of how the virus is able to cause its pathogenic effects is known. To add to this there are two types of F-SFFV which are able to cause either an anaemia or a polycythaemia in susceptible adult mice. Further characterisation of these SFFVs reveals that there are four different strains of the polycythaemia inducing SFFVp, three of which have been previously molecularly cloned (Linemeyer et al., 1980: Yamamoto et al., 1981; Amanua et al., 1983). In the work presented here the fourth strain (Mirand, 1968) was molecularly cloned along with its helper virus. The cloning approach utilised in this study was novel in that the source of the DNA to be cloned (both F-MuLV and SFFVpM) was of extrachromosomal origin whose presence was shown to be quantitatively proportional to the differentiation state and also the differentiation ability of the Friend cells analysed. This phenomenon (first described by Ikawa et al., 1980) was shown to be confined to Friend cells (as compared to the other cells used in this study) and until recently had only been observed in one other system (MMTV infected rat hepatoma cells, Ringold et al., 1978). However recently an extrachromosomal form of a retroviral DNA molecule (HTLV-1, Hiramatsu et al., 1988) has also been identified within HTLV-1 infected human HL60 cells. It was shown by these workers that these extrachromosomal DNA molecules (both linear and circular) were able to reintegrate into the host cell genome upon passage of these cells. In the study described here, no detectable new integration sites could be

detected within the genome of F4-6 cells during the DMSO induction of these cells or indeed over one year of continuous culture (data not shown). However one problem involved in this analysis in F4-6 cells is the lack of a specific probe which only detects integrated F-SFFVpM sequences and not env related endogenous sequences which are present in the DBA/2J genome from which these cells originate thus making detection of new integration site very difficult. This phenomenon could possibly be analysed more easily now, for as described in Chapter 4 of the results, Friend cells tansformed by a neo^{R} SFFVp are now available thus providing a unique hybridisation probe, however it remains to be seen whether extrachromosomal DNA molecules are at all present within these cells.

Both cloned viral entities were biologically active with the F-MuLV being able to cause a lymphatic leukaemia in new born mice only and F-SFFVpM being able to cause an erythroleukaemia in susceptable adult mice, although not with the altered target cell specificity that was hoped. Thus the one aspect of the work presented in this thesis to molecularly clone the Mirand strain of F-SFFVp along with its helper virus was successful although after encountering major difficulties with the SFFVp component. That the FV complex is regularly used in this laboratory means that specific molecular probes are now available for the molecular analysis of SFFVp in the various experimental cell systems exploited here. The molecular clones of the two viruses may also be used to further examine the molecular similarity suggested by Mol and co-workers (1981). Such information (i.e. complete nucleotide analysis of both of the viral genomes) would enable one to determine as to which recombination events were involved in the genesis of the SFFVp genome within this system for these two FV components have remained

together and have thus co-evolved since their isolation. Comparative analysis of the only fully sequenced SFFVp (Clark and Mak, 1983) suggests that SFFVp is generated by a complex sequence of recombinations involving most likely a minimum of three retroviruses, namely an endogenous retrovirus related to AKV, F-MuLV and a virus with xenotropic sequences. The recombination point of xenotropic/ecotropic SFFV *env* sequences appears to be similar to that of the F-MCF virus. The 3' end from the 5' LTR (U5) to the 3' half of the *gag* gene shows more than 95% nucleotide sequence homology between SFFVp and AKV. The remainder of the *gag* region and the non-deleted *pol* of SFFVp has 75-95% homology to both AKV and F-MuLV: More than 90% homology to xenotropic and xenotropic derived MCFV sequences is found from the 3' end of *pol* to the middle of the *env* gene. The 3' end of *env* is 90-100% homologous to F-MuLV.

A second aspect of the work presented here was to develop a biologically active SFFVp containing a dominant selectable marker gene, which up until now has remained somewhat elusive, (Joyner and Bernstein, 1983; Ostertag *et al.*, 1987). The original consideration was to use the molecularly cloned F-SFFVpM for this construct, but because of the difficulties encountered with the molecular cloning of the virus and also for other considerations described earlier the molecular clone of the Lilly and Steeves strain was utilised (Linemeyer *et al.*, 1980).

In this thesis it has been shown that using an MPSV based retroviral vector as a framework then a biologically active F-SFFVp with a dominant selectable marker gene could be constructed and to some extent was even more potent than the unmodified wild type SFFVp. Furthermore this construct demonstrated that only the *env* and U3 sequences from F-SFFVp are required not only to elicit the erythroproliferative disease,

but also for the transformation event leading to the isolation of Friend cells. Previous workers have used deletion mutants and genetically engineered recombinants which had a SFFV based background, thus not disclosing whether other sequences apart from the *env* gene may indeed contribute to the pathogenicity of the virus, (Linemeyer *et al*., 1982; Ruta *et al*., 1983; Machida *et al*., 1984, 1985). The MPSV vector which formed the backbone of the neo^R SFFVp vector has no pathogenic effects *in vivo* (Ostertag *et al*., 1986).

A further construct which differed from the original neo^R SFFVp only in the origin of it's U3 region demonstrated that this region is not responsible for the tropism and pathogenesis of this construct, although the initial kinetics of the primary disease caused by this virus somewhat lagged behind that of the original neo^R SFFVp. Direct nucleotide sequence comparison of these two U3 regions along with other related viruses, show that within the U3 there is considerable nucleotide heterogeneity displayed between the two sequences, (Figure 58) the most noticeable of which is the deletion of one direct repeat and thus one enhancer in the SFFVp sequences. There is also a noticeable nucleotide heterogeneity at the start of the U3 region and also at the boundary of the direct repeats whose sequence is a putative glucocorticoid receptor binding site. In these regions the MPSV U3 is almost identical to that of the Mo-MuLV LTR which has been shown not to alter the biological activity of a recombinant F-SFFVa (Wolff and Ruscetti, 1986).

The reasons for this difference in kinetics is somewhat unclear although effects on transcription levels could be envisaged (either on gene products or on progeny viral RNA) when one considers other systems where tissue/cell tropism and elevated transcription have been shown to

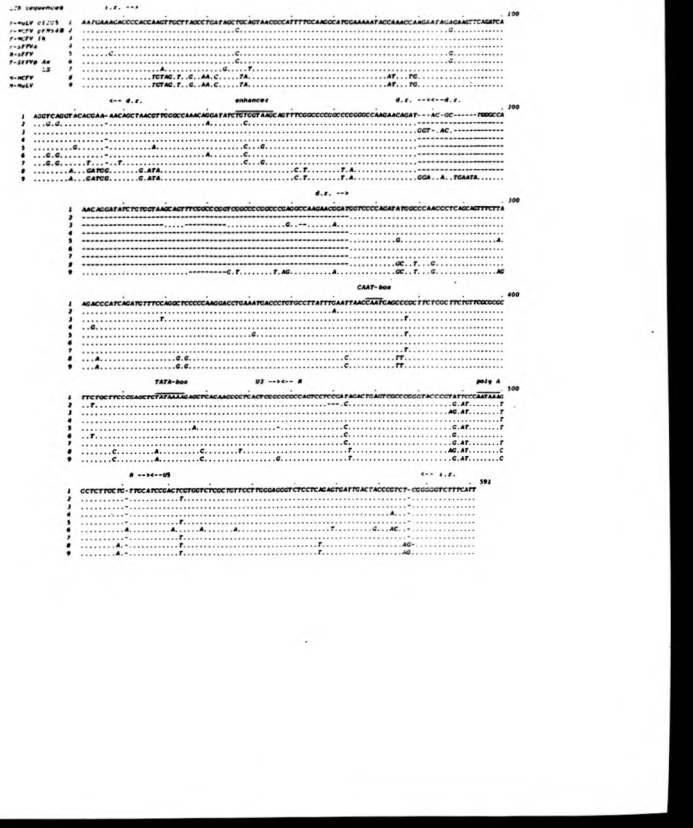


Figure 58. Comparison of LTR nucleotide sequences of SFFVp and related viruses with the LTR sequence of F-MuLV.

Missing nucleotides are indicated by -, inverted repeat sequences are labelled i.r. and direct repeats are labelled d.r. The sequences are taken from;

Koch et al., 1984: F-MuLV 57, F-MCFV,pFM54B;

Adachi et al., 1984: F-MCFV FrMx;

Wolff et al., 1985: F-SFFVa, F-SFFVpLS;

Bestwick et al., 1984: R-SFFV;

Clark and Mak, 1982: SFFVpAx;

Bosselman et al., 1982: M-MCFV c116;

Stacey et al., 1984: MPSV;

Shinnick et al., 1981: Mo-MuLV pmLV1;

Vogt et al., 1985; RMCFV-1.

be dependent upon the cell system.

These vectors may also prove to be useful tools in the dissection of various aspects of the pathogenesis of SFFVp for due to their selectable marker gene (G418 resistance) cells infected with these viruses are easily selected *in vitro* and are also tagged after integration with a sequence which is unique and easily detectable in eukaryotic cells. Thus integration sites and target cells may be analysed as well as SFFVp specific activities (i.e. directed mutagenesis within the *env* gene. R. Friedrich, University of Giessen pers. commun.). A retroviral vector E3 based upon the neo^R SFFVp construct was also constructed so that sequences apart from the F-SFFVp *env* gene could be analysed. This vector may also prove to be useful for high expression of genes within cells of the erythroid lineage or even haemopoietic stem cells.

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Table 10. Spleen erythroid precursor cells of mice infected with neo^R SFFV$_p$ are increased, erythropoietin independent and resistant to genecitin

Virus	Spleen wt. (g)	Benzidine positive CFU-E/10^5 spleen cells			
		(-) Epo		(+) Epo	
		-G418	+G418	-G418	+G418
None	0.113 ± 0.01	18 ± 3	4 ± 1	233 ± 60	10 ± 3
SFFV$_p$M (F4-6)	1.45 ± 0.09	3134 ± 188	450 ± 63	3514 ± 220	403 ± 35
Neo^R SFFV$_p$ (8a+H)	1.68 ± 0.1	3404 ± 361	3149 ± 204	3611 ± 353	3424 ± 300

The assay for CFU-E was performed as described by Fagg and Ostertag. Data are the mean of three independent experiments. The concentration of G418 used was 1.5 mg/ml. Only the benzidine-positive cells were scored as CFU-E in the 48 h assay.