# MOLECULAR CHARACTERISATION OF ENDOGENOUS LOCI RELATED TO JAAGSIEKTE SHEEP RETROVIRUS

by

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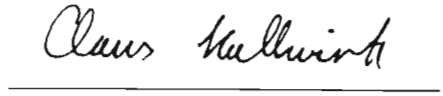
# DECLARATION

This study represents original work by the author and has not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.

The research described in this thesis was carried out in the Department of Virology, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, under the supervision of Dr. D.F. York, and in part at the Cancer Research Institute and Department of Molecular Biology and Biochemistry, University of California, Irvine, under the supervision of Professor H.Y. Fan.



Claus Volker Hallwirth

2007

# DEDICATION

Dedicated to my loving wife, Kumari.

# CONFERENCE PRESENTATIONS

This study has been presented in part at the following congresses:

**Oral**

1. Hallwirth, C.V. & York, D.F. (2002, September). Molecular characterisation of endogenous jaagsiekte sheep retrovirus loci. University of Natal, Faculty of Health Sciences Astra-Zeneca Research Day.

2. Hallwirth, C.V, York, D.F. and Fan, H.Y. (2004, September).Variable regions 1 and 2 (VR1 and VR2) in JSRV *gag* are not responsible for the endogenous JSRV particle release defect. University of KwaZulu-Natal, College of Health Sciences Astra-Zeneca Research Symposium.

**Poster**

1. Hallwirth, C.V., Palmarini, M., Murgia, C., de Oliveira, T., Fan, H., and York, D.F. (2000, May). Isolation and characterization of three full-length JSRV-related endogenous retroviruses from sheep DNA. Cold Spring Harbor Laboratories, Long Island, New York: 2000 Conference on Retroviruses.

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# PUBLICATIONS

The following publications have arisen from the work described in this thesis:

1. Palmarini, M.*, Hallwirth, C.*, York, D., Murgia, C., de Oliveira, T., Spencer, T. and Fan, H. (2000). Molecular cloning and functional analysis of three type D endogenous retroviruses of sheep reveal a different cell tropism from that of the highly related exogenous jaagsiekte sheep retrovirus. *Journal of Virology* 74 (17): 8065-76.

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2. Verwoerd, D.W., Tustin, R.C., Hallwirth, C.V. and York, D.F., 2005. Chapter 59 – Jaagsiekte. Volume 2, pp. 717-732. In: J.A.W. Coetzer and R.C. Tustin (eds), *Infectious Diseases of Livestock, Second Edition*, (Oxford University Press, Southern Africa: Cape Town).

3. Hallwirth C., Maeda N., York D. and Fan H. (2005). Variable regions 1 and 2 (VR1 and VR2) in JSRV *gag* are not responsible for the endogenous JSRV particle release defect. *Virus Genes* 30 (1): 59-68.

4. McGee-Estrada K., Palmarini M., Hallwirth C. and Fan H. (2005). A Moloney murine leukemia virus driven by the jaagsiekte sheep retrovirus enhancers shows enhanced specificity for infectivity in lung epithelial cells. *Virus Genes* 31(3): 257-263.

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# ABBREVIATIONS

| | |
|---|---|
| ALSV | avian sarcoma-leukaemia virus |
| ATII | alveolar type II |
| BAC | bronchioloalveolar carcinoma |
| bp | base pair |
| CA | capsid protein; part of Gag |
| CEF | chicken embryo fibroblast |
| CMV | cytomegalovirus |
| CRI/UCI | Cancer Research Institute, University of California, Irvine |
| CT | cytoplasmic tail |
| $dd_iH_2O$ | distilled, deionised, autoclaved water |
| DEPC | diethyl pyrocarbonate |
| ENTV | enzootic nasal tumour virus |
| ER | endoplasmic reticulum |
| GPI | glycosylphosphatidyl-inositol |
| Grb | growth factor receptor binding protein |
| HA | haemagglutinin |
| Hyal | hyaluronidase |
| IN | integrase enzyme encoded by the retroviral *pol* gene |
| JSRV | jaagsiekte sheep retrovirus |
| LB | Luria-Bertani |
| LTR | long terminal repeat |
| MA | matrix protein; part of Gag |
| MAPK | mitogen-activated protein kinase |

| | |
|---|---|
| MDCK | Madin-Darby canine kidney |
| M-MuLV | Moloney murine leukaemia virus |
| MSD | membrane spanning domain |
| MSP | macrophage stimulating protein |
| NC | nucleocapsid protein; part of Gag |
| nt | nucleotide |
| OPA | ovine pulmonary adenocarcinoma |
| ORF | open reading frame |
| PBS | phosphate-buffered saline |
| pfu | plaque-forming units |
| PrBS | primer binding site |
| PRR | proline rich region |
| PI3K | phosphatidylinositol 3-kinase |
| R | repeat region in retroviral LTR |
| RE | restriction endonuclease |
| RT | reverse transcriptase |
| SRP | signal recognition peptide |
| SU | retroviral envelope surface protein |
| TIGEF | large T-immortalised goat embryo fibroblast |
| TM | retroviral envelope transmembrane protein |
| VR | variable region |

# ETHICS

This entire study was approved by the Ethics Committee of the Nelson R. Mandela School of Medicine, College of Health Sciences, University of KwaZulu-Natal.

# ABSTRACT

The study of retroviruses has been of pivotal significance to the field of biomedical science, where it has provided fundamental insights into the processes underlying both viral and non-viral carcinogenesis. Ovine pulmonary adenocarcinoma (OPA), a contagious lung cancer of sheep and goats, has emerged over the past three decades as an invaluable model of human epithelial cancers. It is one of the very few animal models of retrovirus-induced neoplasia of epithelial tissues, whereas most other such animal models of human cancers pertain to the haematopoietic system. OPA represents a unique, naturally occurring, inducible, outbred animal model of peripheral lung carcinomas, and is caused by a betaretrovirus – jaagsiekte sheep retrovirus (JSRV) – that is receiving increasing attention in the fields of retrovirology and lung cancer research.

JSRV exists in two highly homologous, yet molecularly distinct forms. The first is an exogenous form of the virus that is transmitted horizontally from one animal to another. This form is infectious and the direct cause of OPA. The other is an endogenous form, 15 to 20 proviral copies of which reside benignly in the genome of sheep and are transmitted vertically from one generation to the next. At the time this study commenced, no knowledge existed regarding the underlying pathogenic mechanism by which JSRV causes OPA. Even though the nucleotide sequence of exogenous JSRV had been elucidated seven years earlier, only limited sequence information was available on endogenous JSRVs. With a view towards identifying genetic regions or elements within exogenous JSRV that could potentially be implicated in its pathogenic function, this study began with the cloning of the first three full-length endogenous JSRV loci ever isolated from sheep. The DNA sequences of these full-length endogenous JSRV loci were determined and

comprehensively analysed. Comparison with exogenous JSRV isolates revealed that the two forms of the virus are highly homologous, yet can be consistently distinguished in three short regions within the coding genes. Two of these reside in the *gag* gene, and one at the end of the *env* gene. These regions were named the variable regions (VRs) of sheep betaretroviruses.

The JSRV VR3 in *env* was linked by our collaborators to the virus's ability to transform cells in tissue culture. The effects and biological significance of VR1 and VR2 in *gag* are subtler and more difficult to determine. After identifying these regions, it became the objective of this study to develop relevant molecular tools that could be used to discern the significance of these variable regions *in vivo*, and to characterise these tools *in vitro* to assess their suitability for *in vivo* studies. The development of these tools entailed the design of a novel strategy that was implemented to precisely substitute the endogenous VR1 and VR2 (individually and in combination) into an infectious molecular clone of exogenous JSRV. These chimeric constructs were shown to support retroviral particle release into the supernatant of transiently transfected cells in tissue culture. These particles were confirmed by independent experiments to have arisen specifically from transfection with the chimeric clones. Finally, the particles were shown to be capable of infecting cultured cells and of productively integrating their genomes into those of their host cells, rendering these particles fully competent retroviruses that can be used in the context of *in vivo* studies to determine the biological significance of VR1 and VR2.

This study has made a significant contribution to the further development of the OPA / JSRV model system of human epithelial lung cancers. It has also led to the design of a molecular substitution strategy that can be adapted to introduce any genetic region into a

cloned DNA construct, regardless of the degree – or lack of interrelation – of the two DNA sequences, thereby creating a highly versatile molecular biological tool.

# CHAPTER 1

# GENERAL INTRODUCTION

# AND LITERATURE REVIEW

## 1.1 THE STUDY OF RETROVIRUSES

The discovery and study of retroviruses has been of far-reaching significance in the fields of biology and medicine (Vogt, 1997a). The signet of these viruses lies in their replicative strategy and the associated RNA-dependent DNA polymerase enzyme, reverse transcriptase. The discovery of this enzyme (Baltimore, 1970; Temin & Mizutani, 1970) had tremendous scientific impact by necessitating a revision of the hitherto accepted central dogma of the flow of genetic information from DNA to RNA to protein. Since its discovery, it has become an indispensable tool for the operation of virtually all molecular biology and biotechnology laboratories.

The study of retroviral oncogenesis led to the discovery of viral oncogenes and their cellular origin in the form of proto-oncogenes (Fan, 1994; Rosenberg & Jolicoeur, 1997; Vogt, 1997a). All retrovirally transduced oncogenes were found to be of normal cellular origin, and the common mechanisms involved in retrovirally induced neoplasms extend to tumours lacking a retroviral aetiology – many of the genes that were initially identified as retroviral oncogenes in animal cancer models are now known to be activated in certain types of human cancers (Barbacid, 1987; Bos, 1989; Fan, 1994; Hynes & Stern, 1994; Sawyers, 1992). Even important pathways involved in normal cellular growth and

differentiation were elucidated and continue to be illuminated by studies of retroviral systems (Rosenberg & Jolicoeur, 1997).

In addition to their roles in promoting our understanding of normal and aberrant cellular growth processes and providing us with an invaluable tool in molecular biology, retroviruses hold immense therapeutic potential in the form of transfer vectors for genetic material in gene therapy. The use of retroviral constructs is being clinically pursued or experimentally investigated for gene therapy targeting various tissues and organs, *e.g.* muscle (Fassati & Bresolin, 2000), liver (Kalpana, 1999), the central nervous system (Suhr & Gage, 1999), the haematopoietic system (Cornetta & Fan, 1997) and even stem cells (Havenga *et al.*, 1997), mostly for the treatment of cancers, but also for numerous congenital and acquired diseases. The development and efficacy of such therapeutic approaches relies on a continually more detailed understanding of retroviruses, their induced pathogenesis and their life cycles, as well as the identification of novel, and the characterisation of hitherto relatively unknown, retroviruses.

## 1.2 THE DISEASE: JAAGSIEKTE / OVINE PULMONARY ADENOCARCINOMA

"Jaagsickte", which translates directly to "chasing sickness", is the Afrikaans name originally given to a contagious lung cancer of sheep, since affected animals appeared as though they had been chased. Previously the disease was also referred to as sheep pulmonary adenomatosis or ovine pulmonary carcinoma. As of June 2001, the various research groups working with it agreed on the standardised use of "ovine pulmonary

adenocarcinoma" (OPA) to describe this disease (Fan, 2003). "Ovine" refers to sheep; "pulmonary" relates to the lungs; the prefix "adeno-" means "gland"; and carcinomas are malignant tumours originating in epithelial tissues. OPA was further classified as a bronchioloalveolar carcinoma (BAC) (Nobel & Perk, 1978) in accordance with the previously accepted World Health Organisation classification criteria for tumours of domestic animals (Stunzi *et al.*, 1974).

OPA is a well-differentiated multifocal tumour that originates from the transformation of epithelial alveolar type II (ATII) pneumocytes and the non-ciliated bronchiolar (Clara) cells (Nisbet *et al.*, 1971; Nobel *et al.*, 1969; Nobel & Perk, 1978; Perk *et al.*, 1971b; Perk *et al.*, 1971a). These early reports were recently confirmed when the transformed cell types were proportionally enumerated – 82% were found to be ATII cells, 7% Clara cells and 11% could not be classified owing to insufficient differentiation (Platt *et al.*, 2002). Metastasis to regional pulmonary lymph nodes occurs in 10 to 50 % of cases, depending on the country in which the data originate (DeMartini & York, 1997; Verwoerd, 1996). The cause of this variation has not been established, but might be linked either to differences in breed susceptibility or to different viral strains (Verwoerd, 1996). The transformed cells do not lose their secretory function, and the lung fluid production that is characteristic and clinically diagnostic of OPA probably originates from the tumour cells (DeMartini & York, 1997).

OPA is one of the major infectious diseases of sheep. It displays an extremely variable incidence and prevalence worldwide, occurring in sheep flocks in many countries of Africa, Asia, South America and Europe (DeMartini & York, 1997; Hecht *et al.*, 1996a; Sharp & DeMartini, 2003). In some of these, including South Africa, prevalence is as high

as 20 % in certain flocks and is thus of considerable economic importance, whereas it is infrequently diagnosed in the United States and Canada. OPA was successfully eradicated in Iceland by means of a radical slaughter strategy (Sharp & DeMartini, 2003; York & Querat, 2003), and has never been reported in Australasia and the Falkland Islands (Ortin *et al.*, 1998), even though the large population of Merino sheep in Australia originated from South Africa (Verwoerd, 1996). The most recent region to report OPA for the first time is Patagonia, Argentina, which should have been protected against the introduction of the disease by the animal health barrier that restricts the introduction of live animals or their products into that area (Uzal *et al.*, 2004). The source of OPA infection in Patagonia remains unknown.

Clinical symptoms of OPA manifest once the tumour mass becomes large enough to hamper normal lung function; affected animals develop progressive respiratory distress and usually die within a few weeks of the onset of clinical signs (De las Heras *et al.*, 2003a). The natural incubation period of OPA has been estimated at approximately five to six months, with two- to four-year-old sheep being most commonly affected (DeMartini *et al.*, 1988; DeMartini & York, 1997; Sharp & DeMartini, 2003). Experimentally, however, OPA can be induced within three to four weeks (and even as little as ten days) in newborn lambs, upon intratracheal inoculation with concentrated lung fluid collected from OPA-affected sheep (Sharp *et al.*, 1983). A later study demonstrated that OPA can be induced by inoculation of lambs of ages up to six months, and that the incubation period is longer for lambs inoculated at a higher age (Salvatori *et al.*, 2004).

## 1.3 JAAGSIEKTE SHEEP RETROVIRUS

Jaagsiekte sheep retrovirus (JSRV) is currently the only virus known to induce a naturally occurring lung cancer (Palmarini *et al.*, 2004). JSRV resembles type D and type B oncoviruses in terms of its morphology and genetic organisation (York *et al.*, 1991; York *et al.*, 1992), and is phylogenetically closely related to mouse mammary tumour virus (MMTV) and to a group of human endogenous retroviruses (HERVs) that utilise a lysine (K) tRNA recognition sequence as their primer binding site (PrBS) for second strand synthesis during reverse transcription, hence the designation "HERV-K". Its closest exogenous relative is enzootic nasal tumour virus (ENTV), which causes a nasal adenocarcinoma arising from the transformation of cells in the respiratory and olfactory mucosal glands of sheep and goats (De las Heras *et al.*, 2003b). JSRV exists in two closely related, but molecularly distinct forms. The first is an infectious exogenous retrovirus (henceforth referred to in this manuscript as JSRV), which is transmitted horizontally from one animal to another and is the aetiological agent of OPA (Palmarini *et al.*, 1999a). The second is a group of endogenous retroviral loci that resemble JSRV at the nucleotide level (York *et al.*, 1992), but are transmitted vertically through the germ line of animals in the fashion of Mendelian genes. These endogenous sequences are henceforth referred to as enJSRVs in this manuscript. enJSRVs are widely distributed in ungulates, with 15 to 20 copies of closely related sequences being found in the genomes of all domestic sheep and goats (Hecht *et al.*, 1996b). OPA is associated absolutely with its aetiological agent JSRV (Palmarini *et al.*, 1996a), whereas enJSRVs by themselves are not associated with disease. No sheep population has been identified that lacks enJSRVs, and the possible role of these sequences in disease, as well as their importance to the host organism, has yet to be elucidated.

### 1.3.1 JSRV is the aetiological agent of OPA

Historically, herpesviruses and lentiviruses were considered likely candidates as the aetiological agents of OPA. However, the gradually accumulated evidence implicating a retrovirus (specifically JSRV) became increasingly more convincing. It is only briefly summarised here, as it has been extensively reviewed (DeMartini & York, 1997; Hecht *et al.*, 1996a; Palmarini *et al.*, 1997; Verwoerd, 1996; York & Querat, 2003), and is now largely of historical interest:

- Transmissibility studies showed that the disease could be reproduced by inoculation with cell-free extracts of tumour homogenates or concentrated lung fluid.
- Inocula exhibited a reverse transcriptase (RT) activity with a magnesium ion preference, and the RT level in the inocula was inversely proportional to the incubation period in experimental transmission studies.
- Serological studies showed that both lung fluid and tumour homogenate contained proteins that cross-reacted with antisera to capsids and nucleocapsids of type D and type B retroviruses.
- Retrovirus-like particles were observed by electron microscopy in lung tumours and lung fluid of affected sheep.
- The full genome of a retrovirus (JSRV-SA) isolated from the lungs of an OPA-affected sheep was cloned and sequenced (York *et al.*, 1991; York *et al.*, 1992), and it exhibited a genomic organisation typical of type D and B retroviruses.
- JSRV was found to be consistently, specifically and absolutely associated with OPA (Palmarini *et al.*, 1996a).

The above evidence, however, did not prove that infection with JSRV is sufficient to induce OPA by itself. The possibility existed that JSRV was acting in the capacity of a helper virus, complementing a hitherto undiscovered acutely transforming retrovirus. Further definition of the role played by JSRV in OPA was hampered by the lack of an *in vitro* culture system for the virus. The precise nature of association of JSRV with OPA was not known until Palmarini *et al.* (1999a) constructed an infectious molecular clone derived from an integrated proviral JSRV sequence (called $JSRV_{21}$) isolated from a spontaneous case of OPA. The $JSRV_{21}$ genome was placed under the transcriptional control of the human cytomegalovirus (CMV) immediate early promoter. The promoter was positioned in such a way that the resulting RNA transcript would be very similar to wild-type JSRV RNA, giving rise to the plasmid $pCMV2JS_{21}$. $pCMV2JS_{21}$ was transfected into the highly transfectable human 293T cell line, from which substantial amounts of $JSRV_{21}$ virus were released into the culture supernatant. Concentrated stocks of $JSRV_{21}$, obtained from transfected 293T cells, were used for intratracheal inoculation of newborn lambs. Two of them became infected with OPA (confirmed at the histological, immunological and molecular level), thus proving conclusively, for the first time, that JSRV is both necessary and sufficient to induce OPA.

### 1.3.2 Characterisation of JSRV

Arguably, the first cornerstone in the foundation of JSRV molecular biology research was the elucidation of the complete genomic nucleotide sequence of JSRV (York *et al.*, 1991; York *et al.*, 1992). The sequence was obtained by assembling the sequences of individual cDNAs derived from the purified RNA of a South African isolate, JSRV-SA. The

JSRV-SA genome was found to be 7462 nucleotides (nt) in length, measured from the 5' *R* ("repeat") to the 3' *R* region. Analysis of the JSRV-SA sequence revealed that it contains a primer binding site (PrBS) complementary to the 3' end of a $tRNA^{Lys}_{1,2}$ immediately downstream of the U5 region, just like HERV-K. JSRV-SA exhibited a genomic organisation typical of type D and type B retroviruses, having overlapping open reading frames (ORFs) encoding the structural genes *gag*, *pro*, *pol* and *env*, with a separate *pro*

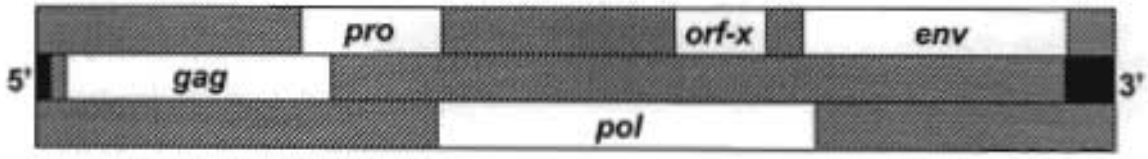



**Figure 1.1 Schematic representation of the genomic organisation and open reading frames of JSRV.** The black boxes on the 5' and 3' ends represent the *R-U5* and *U3-R* regions, respectively, flanking the retroviral RNA genome. Upon integration, they would form the 5' and 3' long terminal repeats of the provirus, each consisting of identical *U3-R-U5* sequences. Adapted from York *et al.* (1992).

reading frame (Fig. 1.1). Retroviral mRNAs are translated into three polyproteins – Gag, Gag-Pro-Pol and Env – which are post-translationally cleaved into mature proteins by the retroviral protease enzyme encoded by the *pro* gene. The *pol* gene encodes the reverse transcriptase (RT) and integrase (IN) enzymes, while the envelope (Env) protein consists of a surface (SU) and a transmembrane (TM) domain (Swanstrom & Wills, 1997). The Gag polyprotein is cleaved minimally into three subunits – MA (matrix protein, which is peripherally, not integrally, associated with the host cell membrane) on the amino-terminal end; CA (capsid protein, which encapsulates the ribonucleoprotein complex containing the genomic RNA); and NC (nucleocapsid, which binds with high affinity to the genomic RNA) on the carboxy-terminal end – although most retroviral Gag proteins contain a segment between MA and CA and one after NC that are often cleaved further into smaller

peptides or proteins (Vogt, 1997b). In JSRV, these have only recently been defined as "p15" (between MA and CA) and "p4" (Murcia *et al.*, 2007).

In addition to the canonical retroviral genes, JSRV-SA had an accessory open reading frame overlapping *pol*, the function and significance of which has yet to be determined. This reading frame was termed "*orf-x*" and was also found in the only other full-length JSRV genomic sequence known at that time – $JSRV_{21}$ (Palmarini *et al.*, 1999a). The *orf-x* region is considered rather unusual. Its predicted polypeptide sequence of 166 amino acids (AA) is very hydrophobic and exhibits a codon usage that is significantly different from that of the *pol* gene. It does not appear to impose any constraints on the codon usage of *pol*, however. Based on the above observations, the prospect of *orf-x* being a coding region was initially considered unlikely (York *et al.*, 1992), a view which was substantiated by a consistent failure to express cloned versions of the gene in bacterial cells (D.F. York, personal communication). Subsequently, three independent publications reported the conservation of *orf-x* in all known exogenous JSRV sequences, as well as in a number of enJSRVs (Bai *et al.*, 1999; Palmarini *et al.*, 1999a; Rosati *et al.*, 2000). The studies concluded that *orf-x* must be a functional gene and that it was not a chance generation in JSRV-SA. There appears to be a weak sequence homology between a 90 amino acid stretch of the deduced sequence of *orf-x* and the mammalian adenosine receptor subtype 3 (Bai *et al.*, 1999; Rosati *et al.*, 2000), a G-protein-coupled receptor that regulates diverse biological events, including cell growth and differentiation. Structural analysis of the putative mRNA transcript of *orf-x* placed it in a category of transcripts that include those coding for oncogenic growth factors, transcriptional factors, and regulatory proteins predicted to be translated poorly.

Rosati *et al.* (2000) concede that the high degree of conservation of *orf-x* might result from the fact that it overlaps *pol*, which needs to be conserved to maintain biological function. It was suggested that *orf-x* encodes a novel, hydrophobic and membrane-bound protein of unknown function. Cousens *et al.* (1999) argued against a potential role of *orf-x* in cellular transformation. Their reasoning was based on the assumption that ENTV utilises the same oncogenic mechanism as JSRV. ENTV has two stop codons in the region homologous to *orf-x*, and therefore does not represent an accessory open reading frame in ENTV (Cousens *et al.*, 1999). Dr Massimo Palmarini (personal communication) argued at the time that the two stop codons observed in the "*orf-x*" region of the published ENTV sequence might merely be an artefact of the sequencing strategy employed by Cousens *et al.* (1999), and that a possibility existed of ENTV exhibiting an uninterrupted *orf-x* reading frame. In fact, when another isolate of ENTV (from an infected goat instead of a sheep) was sequenced, it was found to contain *orf-x* without any stop codons (Ortin *et al.*, 2003).

Phylogenetic analysis showed that JSRV is closely related to Mason-Pfizer monkey virus (MPMV), the type D retroviral prototype, in the Gag, Pro and Pol protein regions, but that the Env proteins of these viruses are not related (York *et al.*, 1992). Conversely, the Env protein encoded by JSRV is homologous in terms of its structural organisation with that of MMTV (the type B retroviral prototype) and HERV-K (also a type B retrovirus), whereas the *gag*, *pro* and *pol* genes of these viruses are not closely related. JSRV was therefore thought to have diverged from the A-B-D evolutionary lineage of retroviruses between the divergence of the B prototype MMTV and the D prototype MPMV. JSRV thus represents a chimera characterised by a type B morphology (as determined by the spikes on the viral envelope, encoded by *env*) and a predominantly type D genome. Another possible explanation of the chimeric nature of JSRV was proposed by Hecht *et al.* (1996b): JSRV

could have arisen as a consequence of homologous recombination between type B and type D retroviruses. In terms of classification, both type D and type B retroviruses belong to the betaretrovirus genus (Büchen-Osmond, 2004).

Phylogenetic analyses based on the U3 region (Bai *et al.*, 1996), as well as *orf-x* and *env* regions (Bai *et al.*, 1999), of various isolates of JSRV indicate that there are two exogenous genotypes of JSRV, the older of the two being endemic to Africa, and the more recent being prevalent in the USA and UK. Furthermore, the African JSRV genotypes group more closely with endogenous JSRV sequences than do the ones from the USA and UK.

### 1.3.3 Cellular distribution and expression of JSRV in the host

The transformed epithelial tumour cells in the alveoli of OPA-affected sheep have been identified as the major sites of proviral JSRV translation and virion assembly (Palmarini *et al.*, 1995). A high level of viral expression is also observed in the mediastinal lymph nodes draining the lungs, and a lower level of expression has been demonstrated in various anatomically dispersed lymphoid tissues (*e.g.* spleen; thymus; bone marrow; mammary lymph node) and peripheral blood mononuclear cells (PBMCs) (Gonzalez *et al.*, 2001; Palmarini *et al.*, 1996b). More specifically, JSRV was found to infect $CD4^+$, $CD8^+$ and B cells, as well as adherent lymphocytes, which exhibited a higher proviral load than the non-adherent cells (Holland *et al.*, 1999). Macrophages in the lungs of affected animals contain the highest proviral burden amongst the non-lung tissues. This type of dispersed viral infection, of both lymphoid and non-lymphoid tissues, is similar to that observed with other betaretroviruses (Palmarini *et al.*, 1996b).

It has not been determined whether lymphoid infection with JSRV precedes infection of the cells where transformation occurs, or if it is a consequence thereof (but see below). Proviral JSRV DNA was detected in lymphoid cells of animals seven days post-inoculation, at which point there were no signs of OPA (Holland *et al.*, 1999). This indicates that lymphoid infection precedes transformation and tumour development. Systemic lymphatic dissemination could also precede transformation. Gonzalez *et al.* (2001) showed that JSRV proviral DNA can readily be detected in the peripheral blood leukocytes of infected, yet non-symptomatic sheep during the pre-clinical phase, and probably prior to transformation of lung epithelial cells, since some sheep showed no discernible lung tumours at necropsy. Similarly, during the recent OPA outbreak in Patagonia, Uzal *et al.* (2004) found a positive reaction using an exogenous JSRV-specific PCR from the lung tissue of a sheep that had not developed any nodules in its lungs at the point of its (OPA-unrelated) death.

Although JSRV evidently infects other cell types, neoplastic transformation is limited to those cells in which JSRV is efficiently expressed, *viz.* the differentiated epithelial cells of the lung (Palmarini *et al.*, 1995; Palmarini *et al.*, 2000a; Palmarini & Fan, 2003). This tight restriction of expression is considered rather unusual for oncogenic retroviruses (Rosenberg & Jolicoeur, 1997). In a recent study JSRV-infected flocks were monitored over an extended period of time (Caporale *et al.*, 2005). It was found that JSRV infection can occur at any point during a sheep's lifetime (perinatally, in the first few months of life, or in adult sheep), and that the majority of JSRV-infected sheep do not develop clinical disease for the duration of their commercial lifespan. The incubation period of naturally acquired OPA, as opposed to OPA induced by concentrated JSRV-containing inocula, was as much as several years. Furthermore, JSRV was found to be more readily detectable in

peripheral blood leucocytes and lymphoid organs during preclinical stages of infection than in the lungs. The investigators suggest a model whereby JSRV infects lymphoid cells in the first instance. These would serve as a reservoir of infectious virus, which only infects type II pneumocytes and/or Clara cells (which have a low replicative index) if these are undergoing active mitosis in response to, for instance, other pulmonary infections.

Whereas the retroviral envelope gene products determine the cell types that can be infected via interaction with specific cellular receptors (Hunter, 1997), enhancer sequences within the LTRs of retroviruses interact with transcription factors of the infected cells (Fan, 1994; Rosenberg & Jolicoeur, 1997). The LTR of JSRV contains enhancer sequences that interact with transcription factors specifically expressed in differentiated epithelial cells of the lungs, rendering efficient expression of this virus lung-specific (McGee-Estrada *et al.*, 2002; Palmarini *et al.*, 1995; Palmarini *et al.*, 2000a; Palmarini & Fan, 2003).

In a recent study contributed to by the author, the LTR enhancer elements of Moloney murine leukaemia virus (M-MuLV) were replaced with those of JSRV, yielding a chimeric M-MuLV / JSRV LTR (McGee-Estrada *et al.*, 2005). Using luciferase reporter assays, the relative activity of this chimeric LTR was shown to be at least five-fold more active in MLE-15 cells (a mouse type II pneumocyte cell line) than in mouse fibroblasts (NIH-3T3 cells) when compared to the wild-type M-MuLV LTR. The JSRV enhancers thus imparted lung cell specificity on the chimeric LTR. What proved to be a more relevant and sensitive measure of retroviral enhancer activity than the reporter assays, however, was the author's contribution to this study. This entailed infectivity studies of M-MuLV viral particles under the control of the chimeric M-MuLV / JSRV LTR, compared to wild-type M-MuLV particles. The relative infectivity of particles under the control of the chimeric LTR was

three to four log values (as opposed to only five-fold) higher in lung epithelial cells than in different non-lung cells, compared to wild-type M-MuLV particles (McGee-Estrada *et al.*, 2005). These experiments clearly demonstrate the high degree of transcriptional specificity of the JSRV LTR enhancer elements for the differentiated cells of the lungs, which could render JSRV a valuable tool in lung gene therapy.

## 1.4 ENDOGENOUS JSRV SEQUENCES

The genomes of all vertebrates have been colonised by endogenous retroviruses during the course of their evolution. They are inherited in much the same fashion as Mendelian genes and for the most part, they are non-infectious and non-pathogenic. Both advantageous and detrimental roles have been suggested for many endogenous retroviruses, but only rarely substantiated experimentally (Boeke & Stoye, 1997; Vogt, 1997a). The existence of numerous endogenous sequences related to JSRV in sheep genomes was first reported by York *et al.* (1992). This finding resulted from molecular probing of sheep genomic DNA with probes based on the sequence elucidated for JSRV-SA. Probes based on the capsid (CA, part of *gag*) region were used to quantify the endogenous genomic sequences related to JSRV; they were found to hybridise to more than 20 bands, both in the tissues of OPA-affected and unaffected sheep (Hecht *et al.*, 1994).

The above findings were extended by Hecht *et al.* (1996b) to demonstrate that probes based on CA and the envelope surface (SU) region hybridised to between 15 and 20 bands in all members of domestic sheep and goats tested. The banding patterns obtained were similar for the different genomes tested, indicating that the integration sites are probably

similarly conserved. Furthermore, the SU and CA probes hybridised under high stringency conditions to samples obtained from six wild *Ovis* and *Capra* genera, implying that the enJSRV loci thus detected must have become fixed in the genomes of sheep and goats prior to the time of their domestication. The *gag* regions were found to be more conserved among sheep and goat enJSRVs than the *env* regions. Recently, using fluorescence *in situ* hybridisation as well as sheep-hamster hybrid cell lines, the chromosomal distribution of enJSRVs was studied for the first time (Carlson *et al.*, 2003). enJSRV proviruses were found to generally be randomly distributed among sheep and goat chromosomes, with the exception of two loci on different chromosomes that contained multiple enJSRV copies, probably in the configuration of tandem repeats. Only two enJSRV chromosomal positions were shared by sheep and goats, indicating that the majority of endogenisation events occurred after speciation of sheep and goats.

Palmarini *et al.* (1996a) reported the consistent presence of a *Sca*I restriction endonuclease (RE) site in the *gag* region of exogenous JSRVs that was absent in enJSRVs. These findings allowed them to design a hemi-nested (hn) PCR protocol that could distinguish between endogenous and exogenous JSRV transcripts on the basis of the differential *Sca*I restriction site in *gag*. The results of these tests showed that transcripts of endogenous JSRV origin are expressed in various sheep tissues, and that the exogenous form of JSRV is exclusively associated with OPA. More recently, enJSRV expression was found to be most abundant in the reproductive system of ewes, and to be upregulated by progesterone (Palmarini *et al.*, 2001a; Spencer *et al.*, 1999). Furthermore, enJSRVs are broadly expressed in the ovine foetus, including tissues involved in the development of the immune system (Sanna *et al.*, 2002). Palmarini *et al.* (1996a) also found the U3 region in the LTRs of enJSRV proviral sequences to contain insertions of 30 and 16 base pairs (bp) relative to

JSRV. This observation led to a far more sensitive hn-PCR, based on the unique differences between the U3 regions of endogenous and exogenous sequences (Palmarini *et al.*, 1996b), facilitating better detection of exogenous JSRV in a background of enJSRV sequences.

Using PCR primers based on the sequence of JSRV, Bai *et al.* (1996) amplified a number of nearly full-length enJSRV fragments from sheep genomes. Based on restriction endonuclease (RE) profiles, they identified six different types of enJSRV loci (Fig. 1.2). Locus 1 and 2 constituted Group I, based on a common *Eco*RI site in *pol* that was not

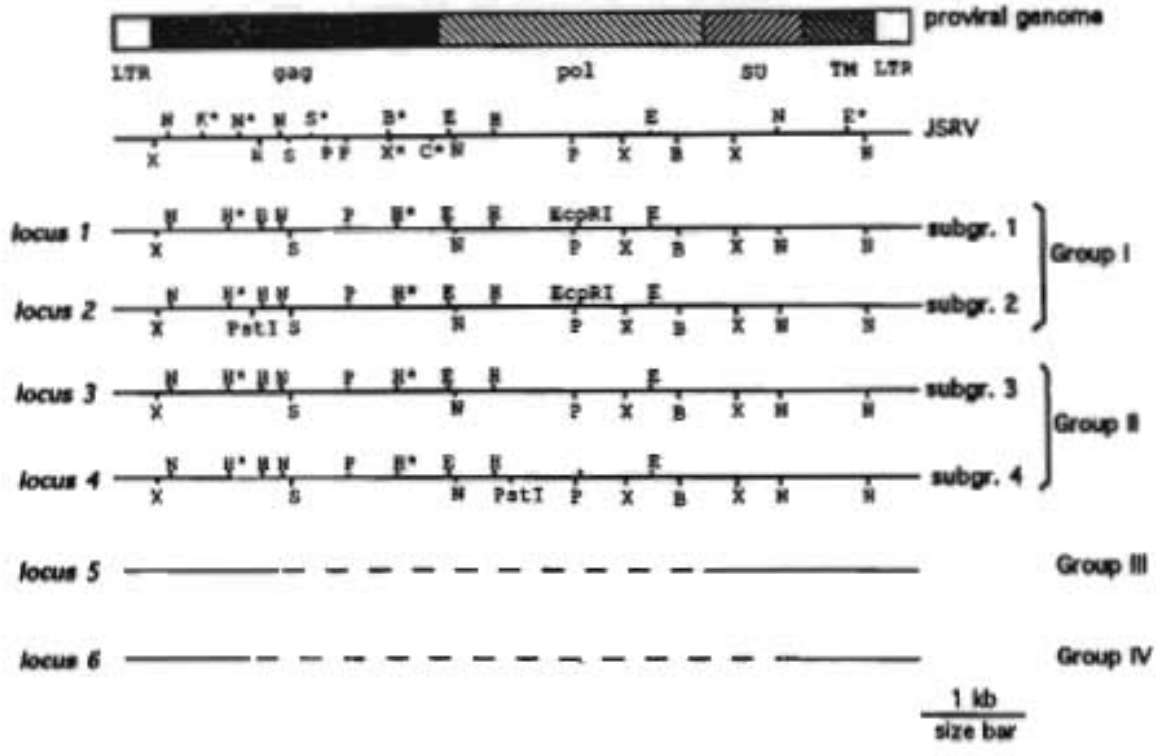



**Figure 1.2 Comparison of restriction profiles of JSRV and enJSRVs** (Bai *et al.*, 1996). Restriction profiles of enJSRVs are aligned with those of JSRV. Sites present only in JSRV or enJSRVs are highlighted with asterisks. Those sites used to distinguish the four subgroups (loci 1 to 4) are indicated by full enzyme names, *Eco*RI and *Pst*I. Dashed lines indicate the gross locations of deletions. Capital letters represent restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; P, *Pst*I; S, *Sca*I; X, *Xho*I. [Figure derived and legend adapted from Bai *et al.* (1996).]

observed in the other loci. A *PstI* site present in the *gag* region of locus 2, but absent in locus 1, distinguished subgroup 1 and subgroup 2 within Group I. For similar reasons, Group II (comprising locus 3 and locus 4) was divided into subgroups 3 and 4. Groups III and IV consisted of loci 5 and 6, respectively, which were characterised by large deletions. No restriction maps were published for Groups III and IV. A 220-bp region comprising part of *gag* was sequenced in 15 enJSRV clones from loci 1 to 6; these sequences exhibited 98% sequence identity amongst each other and an average of 95% identity in the deduced AA sequence with the corresponding JSRV region. Sequence comparisons of parts of the *env* transmembrane (TM) regions of enJSRVs showed that a 500-bp stretch on the 5' end of this region was 96% identical to JSRV at the nucleotide level. However, a 130-bp region of loci 1 to 4 at the 3' end of *env* exhibited only 62% nucleotide and 30% deduced AA identity with the corresponding JSRV region, and was 12 bp shorter. A later study (Bai *et al.*, 1999) confirmed these findings, but the significance of the divergence between the 3' ends of TM in endogenous and exogenous JSRV was not discussed.

## 1.5 INFECTION WITH JSRV IS IMMUNOLOGICALLY SILENT

York *et al.* (1992) pointed out that they had expected their finding of numerous endogenous JSRV-related sequences in the genome of healthy sheep, based on the observation that there was no detectable antibody response to infection with JSRV in sheep (DeMartini *et al.*, 1988; Sharp & Herring, 1983). York *et al.* (1992) speculated that the expression of endogenous JSRV antigens, closely related to those of JSRV, in the neonatal period during ontogeny would lead to the depletion of JSRV antigen-reactive T and B

lymphocyte populations. This would induce a state of immune tolerance with respect to JSRV antigens, both of endogenous and exogenous origin, so that no antibody immune response is launched upon infection with JSRV.

The earlier findings regarding the lack of antibody response to JSRV (and ENTV) were supposedly improved upon by two reports of the detection of antibodies directed against a recombinant major core protein (p27) of MPMV coupled to glutathione-S-transferase (GST) (Kwang *et al.*, 1995; Rosati *et al.*, 1995). These antibody responses were detected in sheep with OPA and goats with ENT. The design of the MPMV p27-GST fusion protein for the detection of infection with JSRV or ENTV was based on earlier findings that MPMV p27 cross-reacts with the capsid antigen of JSRV (Sharp & Herring, 1983) and ENTV (De las Heras *et al.*, 1993). Rosati *et al.* (1995) claimed that neither sera from ENT-affected nor unaffected goats reacted with the GST moiety alone, although they did not show results to substantiate this. Kwang *et al.* (1995) found the MPMV p27-GST fusion protein to react with all OPA-positive control sera but not with any of the negative control sera. They developed an ELISA for OPA serodiagnosis and applied it to US and European sheep flocks. Based on a number of sheep that tested positive for antigens specific for MPMV p27-GST, they concluded that there is a high prevalence of subclinical infection with JSRV.

The above findings were dismissed by Ortín *et al.* (1998) as having been based on non-specific reactions. Ortín *et al.* (1998), using a recombinant JSRV-CA protein coupled to GST, also detected positive reactions in the sera of OPA-affected sheep. However, these positive reactions were not limited to infected sheep; they were also detected in non-affected sheep, as well as sheep from Australia and the Falkland Islands (where OPA has

never been reported). Ortín *et al.* (1998) showed that the reactivity in all sera could be completely abolished by absorption with a lysate containing GST, indicating that the positive reactions demonstrated by Kwang *et al.* (1995) and Rosati *et al.* (1995) were likely to be directed against the helminth parasite-derived GST, and not the JSRV or ENTV capsid antigens. The positive reactions were thus indicative of infections by parasites rather than JSRV or ENTV. These findings therefore confirmed the earlier observations regarding the absence of a specific humoral immune response directed against JSRV. Further support for this was rendered by way of explanation for this phenomenon: The thymus and lympoid tissues associated with the small intestine of lamb foeti were shown to express enJSRV transcripts (Spencer *et al.*, 2003). These tissues are partly responsible for the development of the immune system in sheep, and expression of enJSRV transcripts would lead to their being recognised as "self". The high degree of homology between enJSRVs and JSRV / ENTV would consequently lead to immune tolerance of the latter, as already speculated by York *et al.* (1992).

Recent studies investigated the non-humoral immune response in the lungs of OPA-affected animals and found evidence of a strong local infiltration of macrophages during OPA development, with concomitant release of IFN-γ (Summers *et al.*, 2005). Also, even though infection with JSRV is still considered immunologically silent owing most probably to central tolerance as a result of *in utero* expression of endogenous JSRV sequences, humoral immune responses to vaccination with JSRV capsid and nucleocapsid proteins were generated when these proteins were administered subcutaneously and intramuscularly to sheep with complete Freund's adjuvant (Summers *et al.*, 2006). This finding promises the potential development of a protective vaccine in future.

## 1.6 ONCOGENIC POTENTIAL OF JSRV

The mechanism of transformation leading to OPA has still not been fully elucidated (however, see 3.4.2 for a critical appraisal of recent *in vitro* work to determine this mechanism), but the possible means by which JSRV could cause neoplasia were reviewed by Hecht *et al.* (1996a). Owing to the discovery by Palmarini *et al.* (1999a) that the $JSRV_{21}$ provirus had integrated into part of the sheep genome that did not align with any known cellular sequences (including proto-oncogenes), the possibility of insertional mutagenesis being the transforming mechanism was strongly reduced. Even at this stage, however, insertional activation has not been eliminated as a possible contributor towards OPA, especially in view of the fact that the analysis of 70 different JSRV integration sites in OPA did identify at least one common integration site near a mitogen-activated protein kinase-encoding gene (Cousens *et al.*, 2004). The remaining integration sites, however, displayed an apparently random distribution. Furthermore, since the $JSRV_{21}$ clone was able to induce OPA by itself, in the same time frame as field isolates, JSRV is not a helper virus for an acutely transforming retrovirus. It can therefore be concluded that the genome of JSRV itself is most likely to harbour its oncogenic potential.

This leaves three possible alternatives suggested by Hecht *et al.* (1996a). Firstly, even though JSRV does not encode any sequences known to be associated with oncogenesis, one of its protein products may still induce cellular transformation. Secondly, one of the viral products may act as a transcription factor to stimulate a cellular oncogene. This type of trans-activation is usually associated with a longer time course than the one observed in experimentally induced OPA, but JSRV may harbour a more potent activator than those found thus far. The third and remaining alternative suggested by Hecht *et al.* (1996a) is

that the binding of the type II pulmonary epithelial cell receptors by their retroviral ligands could result in signal transduction, thereby stimulating the production of growth factors, followed by cell proliferation and subsequent neoplasia.

## 1.7 HUMAN BRONCHIOLOALVEOLAR CARCINOMA

Lung cancer represents the leading cause of cancer mortality worldwide, in both men and women (Boring *et al.*, 1994). Bronchioloalveolar carcinoma (BAC, a subtype of pulmonary adenocarcinoma) in humans resembles OPA at the ultrastructural and histopathological level (Barsky *et al.*, 1994). Like OPA, it originates from the transformation of Clara cells and type II pneumocytes (Clayton, 1986; Yesner, 1993; Yesner & Carter, 1982), and is equally multifocal in nature (Barsky *et al.*, 1994). OPA has long been considered the only outbred animal model of human adenocarcinomas of the lung, and of BAC in particular (Nobel & Perk, 1978; Palmarini & Fan, 2001; Perk & Hod, 1982).

BAC differs from other forms of lung cancer in a number of respects. Firstly, a far greater proportion of BAC patients are non-smokers than in other types of lung cancer (Barsky *et al.*, 1994; De Frutos Arribas *et al.*, 1996; Fujimoto *et al.*, 1999; Read *et al.*, 2004). The incidence of cigarette smoking in BAC patients does not appear to differ significantly from the population average in several studies, meaning that – depending on the time frame and geographic location of the studies – at least 30% and as many as 65% of BAC patients are non-smokers. Secondly, there is a proportionally higher incidence of BAC in women than in other types of lung cancer (Barsky *et al.*, 1994; Fujimoto *et al.*, 1999; Read *et al.*, 2004).

During a retrospective study of 1527 cases of BAC conducted by Barsky *et al.* (1994) at the UCLA School of Medicine, covering the 35-year period from 1955 to 1990, a male-to-female ratio of around unity was recorded. The overall male-to-female ratio of lung cancer in 1990 was 3:1. Thirdly, Barsky *et al.* (1994) found that the mean age of patients with BAC was lower than that of non-BAC pulmonary adenocarcinoma patients.

The proportional incidence of BAC relative to other forms of lung cancer is frequently reported as having risen sharply over the past four decades (Auerbach & Garfinkel, 1991; Barkley & Green, 1996; Barsky *et al.*, 1994; Ikeda *et al.*, 1991), and it appears to account for most of the observed increase in pulmonary adenocarcinomas (of which BAC is a subtype). For instance, whereas in 1955 BAC represented less than 5% of total lung cancers as reported in the study by Barsky *et al.* (1994), it accounted for 24% in 1990, making it the most frequently diagnosed form of lung cancer. Similar findings were reported in Japan (Ikeda *et al.*, 1991). However, BAC is also still frequently cited as accounting for approximately 3% of lung cancer diagnoses, as was the case several decades ago. There are recent reports to substantiate this claimed incidence. Fujimoto *et al.* (1999) as well as Read *et al.* (2004) report that BAC rates have remained fairly constant, accounting for about 3% of total pulmonary malignancies. It should be noted that these reports do not necessarily contradict the findings of those groups which have found significant increases in the incidence of BAC in their study cohorts. The apparent discrepancy can be taken to mean that the incidence of BAC has increased in some geographical clusters across the world, whereas it has remained unchanged elsewhere.

Barsky *et al.* (1994) refer to the similarities between OPA and BAC, and suggest that a possible viral aetiology for BAC be considered, based on the multifocality of the disease,

since viral-induced neoplasms tend to be multifocal and multiclonal. The findings of a dramatic increase in the incidence of BAC (at least in some centres), a younger age distribution (being indicative of a risk factor different from those in other lung tumours), a sex ratio close to unity and the rather weak link to cigarette smoking substantiate their hypothesis of BAC possibly being a virus-initiated or -promoted disease entity. Barkley & Green (1996) cite a further nine references that have postulated a viral aetiology for BAC. They support the suggestion that BAC is possibly a virally mediated neoplasm. The apparent geographical clustering of high / increasing incidence of BAC would also be in keeping with an infectious aetiology of at least a subset of BACs.

The similarities between OPA and BAC tempt one to speculate about a possible involvement of a hitherto unknown JSRV-related retrovirus in the aetiology of BAC. Indeed, studies have been undertaken with the aim of investigating such a link, yet their findings are inconsistent. A published preliminary abstract of a conference presentation (O'Connell *et al.*, 1998) indicated that a well-characterised rabbit polyclonal antiserum to the JSRV major capsid protein (CA) (Palmarini *et al.*, 1995) was able to detect immunoreactive antigens in the type II pneumocytes and Clara cells of just over half of BAC sections tested, and in a lower percentage of peripheral adenocarcinomas. O'Connell *et al.* (1998) also reported that JSRV-CA positivity in BAC had increased over the past three decades. Following this preliminary report was a publication, by the same investigators, in which the sample numbers tested had been substantially increased to 249 human lung tumours, as well as numerous non-lung adenocarcinomas, non-tumour lung lesions, and normal tissues (De las Heras *et al.*, 2000). Of the BACs, 30% were positive for JSRV-related CA, as were 26% of lung adenocarcinomas, whereas other samples tested were negative.

These results indicated the possible involvement of a JSRV-related retroviral entity in at least some human pulmonary adenocarcinomas. Two subsequent reports (Hiatt & Highsmith, 2002; Yousem *et al.*, 2001) attempted to refute these findings by showing that the BAC sections in their studies (numbering only 26 and 24, respectively) were negative for JSRV DNA (by PCR) and RNA (by RT-PCR), using undisclosed primer sequences for amplification. Since the publication of these reports, the author has maintained that such studies would have been considerably more meaningful on BAC samples that tested positive for JSRV-CA than on immunologically uncharacterised samples, as was the case in the studies by Yousem *et al.* (2001) and Hiatt & Highsmith (2002). Furthermore, the author disagrees with the claim by Yousem *et al.* (2001), that their molecular assessment represents a superior means of evaluating the presence of JSRV. Whereas this statement might be true when pertaining to JSRV sequences specifically, PCR and RT-PCR would be less suitable for the detection of JSRV-related viruses than immunological assays. This is aptly demonstrated by the ability to detect JSRV using antibodies directed against the capsid protein of MPMV or MMTV (Sharp & Herring, 1983). The nucleotide homology of these viruses would not have sufficed for mutual detection by PCR or RT-PCR. This line of reasoning, maintained by the author since the report by Yousem *et al.* (2001), has recently been supported by a review that relates human BAC to OPA (Mornex *et al.*, 2003).

## 1.8 RATIONALE OF STUDY

Carcinomas (cancers arising in epithelial tissues) are relatively rare in animals compared to humans and most animal models of human cancers pertain to those of the haematopoietic

system (Cremer & Gruber, 1992; Hecht *et al.*, 1996a; Rosenberg & Jolicoeur, 1997). OPA is amongst the only naturally occurring, outbred animal models of carcinoma, and represents a unique cancer model system of differentiated epithelial cells of the lung. Since the cause of OPA – jaagsiekte sheep retrovirus – has been established, and the endpoint of disease has been well characterised, this model system provides the opportunity of dissecting the multi-step cellular and molecular processes that lead from infection to tumour development. In this way, the OPA model is superior to the spontaneously occurring feline bronchioloalveolar carcinoma (Grossman *et al.*, 2002), since it can be readily induced via infection with JSRV. Even though a retroviral aetiology for human BAC has not been firmly established, its operative mechanism of transformation – even if triggered differently – is likely to share some pathways with OPA (Grossman *et al.*, 2002; Rosenberg, 2001). The identification of shared pathways would provide potential targets for both intervention and, ultimately, measures for the prevention of BAC, pulmonary adenocarcinomas, and other epithelial cancers.

Dissecting the mechanism of pathogenesis employed by JSRV necessitates the detailed molecular and cellular characterisation of this virus. No information on the mechanism of JSRV pathogenesis was available at the commencement of this study; in fact, the infectious molecular clone $JSRV_{21}$ (Palmarini *et al.*, 1999a) was not even available. This made the selection of regions within JSRV to study for their potential roles in pathogenesis very difficult. The aim of the first part of this study was to identify genetic regions or elements that could be used to distinguish exogenous from endogenous JSRVs. Only limited PCR-derived nucleotide sequence data had been obtained from the non-pathogenic endogenous JSRVs. It was decided that the first step towards the identification of regions to focus on should be a comprehensive comparison between endogenous non-pathogenic and

exogenous pathogenic isolates of JSRV. It was hypothesised that the nucleotide sequence comparison of several full-length endogenous JSRV genomes (as opposed to mere PCR fragments) with exogenous JSRV would reveal characteristics at the genetic level that are consistently different in exogenous *vs.* endogenous JSRVs and could therefore be potentially implicated in the pathogenesis of exogenous JSRV. Chapter 2 of this thesis describes the characterisation of three endogenous JSRV clones and the identification of so-called variable regions that consistently distinguish exogenous pathogenic from endogenous non-pathogenic JSRVs.

# CHAPTER 2

# CLONING, SEQUENCING AND ANALYSIS OF FULL-LENGTH ENDOGENOUS LOCI RELATED TO JAAGSIEKTE SHEEP RETROVIRUS

## 2.1 INTRODUCTION

The author's work that is described in this manuscript was preceded by two series of experiments, which were instrumental in paving the way towards the design of the current study. For this reason, parts of these two series of experiments will be described briefly, focussing on those aspects that impacted on the author's subsequent research.

### 2.1.1 Full genome PCR approach

As part of an undergraduate vacation project, the author spent seven weeks in the molecular section of the Department of Virology in December 1996 and January 1997, seeking to gain some practical research experience and learn techniques relevant to his undergraduate degree. During this period, he pursued two goals. The first was to answer the important question raised by Dr. D.W. Verwoerd in a guest editorial in the *British Veterinary Journal*, namely whether or not sheep from Australia (where OPA does not occur) also harbour JSRV-related endogenous proviruses (Verwoerd, 1996). The second goal was to PCR amplify the largest possible enJSRV fragment from sheep genomic DNA.

The author was provided with muscle tissue of three different Australian sheep, from which he isolated high molecular weight genomic DNA following a FastPrep SPIN (BIO 101 Systems) protocol. Using a primer pair designed in-house for the PCR amplification of the p26 / capsid region of the JSRV *gag* gene, the target was successfully detected in all Australian sheep DNA samples, as well as in the positive controls. This demonstrated, for the first time, the existence of endogenous proviruses related to JSRV in Australian sheep. This result was expected in view of the fact that even some wild relatives of sheep and goats had been shown, though only by molecular hybridisation, to harbour endogenous JSRV-related sequences (Hecht *et al.*, 1996b), but the question had not previously been formally addressed.

Long-range PCR using the Expand High Fidelity PCR System (Boehringer Mannheim) was employed according to the manufacturer's recommendations in an endeavour to amplify large enJSRV products. The primers used for this were all based on the exogenous JSRV-SA sequence (York *et al.*, 1992). Attempts to amplify near full-length enJSRV sequences using primers JB1 and JB2 (Bai *et al.*, 1996) failed, presumably because they could anneal in the same LTR, and such short amplification would have out-competed the possible amplification of full-length sequences. The replacement of primer JB-1 with P-I (Palmarini *et al.*, 1996b) did not lead to successful amplification either, presumably for the same reasons.

It was subsequently decided to target only approximately half the enJSRV genome in each reaction. To this end, primer pairs P-I + OrfX-2 and OrfX-1 + JB-2 were used for the 5' and the 3' halves, respectively. [See Appendix III for annealing position of primers referred to in this thesis.] Following optimisation of PCR conditions, the 3' half (~ 4 kb) of

an endogenous JSRV locus was successfully amplified and cloned into a pMOS-*Blue* T-vector (Amersham). At a later point, the 5' half of an enJSRV locus was also amplified (although not by the author), and both clones were sequenced. However, this approach could give no indication whether the 5' and the 3' halves that had been amplified were each derived from the same enJSRV locus.

### 2.1.2 Screening of sheep genomic library

Dr. Denis York dedicated time during his sabbatical leave at the end of 1997 and the beginning of 1998 to the endeavour of isolating truly full-length endogenous JSRV loci. This work was conducted in the laboratories of the Cancer Research Institute at the University of California, Irvine (henceforth abbreviated to CRI/UCI), where our collaborators were concurrently engaged in the isolation of a full-length exogenous provirus of infectiously integrated JSRV (Palmarini *et al.*, 1999a). To this end, they had already constructed a lambda phage genomic DNA library (using Lambda DASH II Vector and Gigapack III Gold Packaging Extracts from Stratagene) from the lung tumour of a sheep with naturally acquired OPA. The construction of this library had been based on the digestion of DNA from the sheep lung tumour with the RE enzyme *Xba*I, which, according to the only available exogenous JSRV sequence at the time (York *et al.*, 1992), was believed not to cut within JSRV DNA.

The above phage library of approximately $7.5 \times 10^5$ plaque-forming units (pfu) was divided into 15 sub-libraries, each of which was independently amplified by re-infection of *Escherichia coli* XL1-Blue MRA cells (Stratagene). DNA was extracted from aliquots of

each of the sub-libraries and screened for the presence of exogenous JSRV using the exogenous-specific hemi-nested U3 PCR of Palmarini *et al.* (1996b). Those sub-libraries negative for exogenous JSRV were further screened for the presence of enJSRVs, employing the following steps:

- Sub-libraries were plated on to bacterial agar plates.
- Three plaque lifts were performed from each plate, by use of nylon membranes.
- The phages were lysed on the nylon membranes and the membranes were baked to fix the DNA they contained on to the membranes.
- Replica DNA-containing nylon membranes were hybridised with $^{32}$P-labelled JSRV *gag*-specific or *env*-specific probes.
- Those primary plaques that were positive for both probes potentially contained full-length JSRV sequences (since *gag* and *env* are on opposite sides of the retroviral genome) and were consequently picked and re-plated on bacterial lawns.
- Resulting plaques were screened with *gag* and enJSRV LTR probes.
- The positive plaques were amplified for DNA extraction, and the DNA once again tested for the absence of exogenous JSRV using the exogenous-specific hemi-nested U3 PCR of Palmarini *et al.* (1996b).
- Further testing involved the PCR amplification of a portion of *gag* that would contain the exogenous-specific *Sca*I site, and *Sca*I digestion of these PCR products indicated that this site was absent.

On the basis of the above screening process, three recombinant phages containing distinct enJSRV loci were identified. These were used for large-scale phage preparations, phage purification and DNA extraction. The DNA was digested with *Xba*I and ~12 kb bands

positive for JSRV *gag* and *env* by Southern blotting were excised from gels and ligated into a pBluescript plasmid vector (Stratagene). This gave rise to the plasmid clones penJS5F16, penJS56A1 and penJS59A1, which were brought back to the Department of Virology to be used by the author for his research project.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Description of general laboratory practice employed

- All bacterial cultures were grown at 37°C unless otherwise stipulated.
- Aseptic technique was used during all bacterial work.
- All solutions prepared were autoclaved unless otherwise specified.
- Water used, unless otherwise stipulated, was double-distilled, deionised, autoclaved water. This is abbreviated to $dd_iH_2O$ for the remainder of the manuscript.
- The names of suppliers and manufacturers used in this manuscript are those that were applicable at the time of purchase of any product named, even if the company names changed at a later point. For instance, Expand PCR systems were purchased first from Boehringer Mannheim and later from Roche.

### 2.2.2 Preparation of chemically competent *Escherichia coli* cells

Chemically competent *E. coli* cells were prepared according to an adaptation of the method taught in the Department of Genetics, (formerly) University of Natal, Pietermaritzburg, in the years 1995 to 1997. The procedure is based on established methods (Sambrook *et al.*, 1989) and was modified only to suit our laboratory conditions.

1. A single colony of *E. coli* was inoculated into 5 ml Luria-Bertani (LB) medium and grown overnight with vigorous shaking (~250 rpm).

2. Two millilitres of the overnight culture were inoculated into 200 ml LB medium in a 1 l flask. Prior to addition of the overnight culture, 1 ml LB was withdrawn as a blank for the spectrophotometer (Beckman DU600).

3. The 200 ml culture was grown with vigorous shaking and 1 ml fractions withdrawn for spectrophotometric analysis until the $A_{590}$ reached 0.375 (not greater than 0.4).

4. The culture was dispensed into 4 × 50 ml pre-chilled sterile centrifuge tubes. The cells were left on ice for 10 min to cool and were kept cold for the remainder of the procedure.

5. The cells were pelleted at 3,000 rpm for 5 min at 4°C.

6. The supernatant was poured off and each cell pellet was resuspended in 10 ml of cold $CaCl_2$ solution on ice.

7. The cells were pelleted at 2,500 rpm for 5 min at 4°C and the supernatant discarded.

8.* Each cell pellet was once again resuspended in 10 ml of cold $CaCl_2$ solution on ice. The resuspended cells were pooled and kept on ice for 30 min.

9. The cells were pelleted at 2,500 rpm for 5 min at 4°C and the supernatant discarded.

10. The pellet was resuspended in 8 ml ice-cold $CaCl_2$ solution containing 15% glycerol.

11. The cells were dispensed into labelled pre-chilled sterile microcentrifuge tubes in 250 µl aliquots.

12. The cells were frozen immediately in a dry ice / ethanol bath and stored at –70°C.

* The author introduced the pooling of the cells in this step, both for convenience and to assure even cell concentrations in subsequent steps.

Steps 11 and 12 above were later modified by the author as follows:

- The aluminium block that accommodates 1.5 and 2.0 ml microcentrifuge tubes was removed from a laboratory heating device.
- Inside a cooler box, this block was packed in crushed dry ice.
- Sterile microcentrifuge tubes were labelled and placed into the holes of the aluminium block.
- The cells were aliquotted into the microcentrifuge tubes in the aluminium block and subsequently stored at –70°C.

The above modification has several advantages:

a. The procedure takes less time, because aliquotting of the full 8 ml can be completed without the need to take batches of aliquotted cells to a –70°C freezer, nor do microcentrifuge tubes have to be inserted into a dry ice / ethanol bath.

b. The cells are frozen as soon as they are pipetted into the microcentrifuge tubes, thus optimising their competence.

c. The labels do not come off, as they are apt to do in the dry ice / ethanol bath.

**Solutions required:**

LB medium (1 l)

10 g tryptone

5 g yeast extract

10 g NaCl

Dissolved in 800 ml distilled water, then made up to 1 l with distilled water and dispensed into five aliquots of 200 ml in 1 l flasks prior to autoclaving.

$CaCl_2$ solution

60 mM $CaCl_2$

10 mM Tris buffer, pH 8

$CaCl_2$ solution containing 15% glycerol

As above, except that glycerol was added to a final concentration of 15% before the final volume was adjusted. Addition of glycerol permits long-term storage of competent cells at -70°C.

### 2.2.3 Transformation of competent *E. coli* cells

A general procedure for the transformation of chemically competent *E. coli* cells is given below. This was adapted, in terms of heat shock times and temperatures, depending on the host strain used. When competent cells were purchased (as opposed to being made competent in-house), the suppliers' protocols were followed.

1. Competent cells were thawed on ice and divided into 100 µl aliquots in sterile microcentrifuge tubes.
2. Depending on the type of transformation, either half a ligation mixture (typically 10 µl) or 100 pg to 10 ng of plasmid DNA (depending on the host strain used) was added to the competent cells and mixed by finger tapping. To assess transformation efficiency, an uncut plasmid vector (2 ng) was frequently used as a positive control.
3. This competent cell / DNA mixture was left on ice for 30 min.
4. The cells were heat shocked in a 42°C water bath, for 45 sec (strain JM105) or 2 min (strains DH5a and GM2163).

5. One millilitre of pre-warmed (37°C) SOC (Stratagene; for DH5a and GM2163) or LB medium (for JM105) not containing any selective antibiotic was added, and cells were left to incubate at 37°C for one hour.

6. Aliquots of transformation culture were plated on agar plates containing one or more antibiotics appropriate for the selection of plasmids used for the transformation. In the case of transformation with ligation mixtures, a 90 µl aliquot was usually plated on one agar plate. The remaining cells were pelleted and resuspended in ~100 µl of medium, and plated on a second agar plate. Where colour selection was used, agar plates also contained 80 µg/ml X-gal and 0.1 mM IPTG.

7. When the plates were dry, they were inverted and left in a 37°C incubator overnight.

### 2.2.4 Plasmid isolations

#### 2.2.4.1 MaxiPrep

All large-scale plasmid preparations were performed using either the CONCERT High Purity Plasmid MaxiPrep Purification Kit (Gibco-BRL, Life Technologies) or the Plasmid Maxi Kit (Qiagen), according to the manufacturers' protocols. The former kit was discontinued and last used by the author in 2001. The use of the Qiagen kit was more convenient owing to the availability in the department of the so-called QIArack, in which the QIAGEN-tips 500 could be placed during the column binding, washing and elution

steps. The Gibco-BRL kits, on the other hand, yielded more consistent results in terms of DNA yield and purity from saturated 100 ml cultures and had fewer faulty columns.

#### 2.2.4.2 MiniPrep

The QIAprep Spin MiniPrep Kit (Qiagen) was used for small-scale preparation of high-purity plasmid DNA, essentially according to the manufacturer's instructions pertaining to the use of a microcentrifuge. It was optimised for purification of high-copy number plasmid DNA, which was expanded by overnight broth culture of transformed *E. coli* strains, comprising 1-5 ml LB medium. Prior to the procedure being performed, the following manufacturer's guidelines were employed:

RNase A solution was added to buffer P1.
Buffer PE required the addition of 96-100% ethanol.
Buffers P2 and N3 were checked for salt precipitation. If salt crystals were evident, they were dissolved by incubation at 37°C.
All centrifugation steps were carried out at maximum speed ($\geq 10{,}000 \times g$) in a table top microcentrifuge.

Procedure:

- Bacterial cells (in pellet form) were resuspended in a 250 µl volume of buffer P1. The resulting homogeneous suspension of cells was transferred to a 1.5 ml microcentrifuge tube.

- A 250 µl volume of buffer P2 was added to the cell suspension. The tube was gently inverted 4-6 times to mix the contents. Vortexing was not recommended as the force would cause genomic DNA shearing to occur. The lysis was continued for up to five minutes, by inverting the tube until the solution became visibly viscous.
- Buffer N3 was added to the lysate at a volume of 350 µl. The contents were gently but thoroughly mixed to prevent salt precipitation.
- The solution was centrifuged at maximum speed for ten minutes.
- The supernatant was collected and transferred to a QIAprep column attached to a collection tube.
- The QIAprep column containing the lysate was centrifuged for one minute at maximum speed. The supernatant was discarded.
- The plasmid DNA attached to the QIAprep spin column was washed with 500 µl buffer PB and centrifuged for one minute at maximum speed since mostly *E. coli* JM strains were used.
- The filter tube was disconnected and the flow-through supernatant was discarded.
- A further wash was carried out using buffer PE at a volume of 750 µl. The tube was centrifuged for one minute at maximum speed.
- The supernatant was disposed of and the filter tube was re-connected to the collection tube. The tube was centrifuged for a further minute to collect residual wash buffer at the bottom of the collection tube. This was an important additional step as the wash buffer contained ethanol, which would have inhibited downstream reactions if not completely removed.
- At this point, the collection tube was discarded and the QIAprep column was placed into a sterile 1.5 ml microcentrifuge tube. A 50 µl volume of buffer EB (10 mM Tris-Cl, pH 8.5) was added to the filter tube. The tube contents were allowed to stand at

room temperature for one minute to facilitate the elution process, before being subjected to centrifugation for one minute at maximum speed.

### 2.2.5 DNA quantitation

The DNA utilised as template in each PCR assay or sequencing reaction was quantitated using a Beckman DU 530 Spectrophotometer by measuring the absorbance of the purified DNA at 260 nm. The DNA concentration was calculated by the spectrophotometer using the following parameters: cell path length (mm) of 10 and double-stranded DNA absorption factor of 50. The DNA concentration was determined from the absorbance value at 260 nm. The instrument was fitted with a sipper accessory that drew the fluid into a chamber of a path length equivalent to that of a quartz cuvette.

Procedure:

- A 1:100 dilution of each sample was made and placed into a micro-centrifuge tube.
- It was vortexed thoroughly.
- An 800 µl volume of distilled water, which served as the blank, was taken up by the sipper (all DNA samples were reconstituted in $dd_iH_2O$).
- The absorbance at 260 nm was displayed as 0.00.
- Each absorbance at 260 nm of each sample was measured using the same procedure. There was no washing of the sipper between samples.
- The ratio ($A_{260}$ / $A_{280}$) was obtained for each DNA sample to detect protein contamination and determine sample purity.

- After all samples had been spectrophotometrically read, the sipper was washed with distilled water.

### 2.2.6 Polymerase chain reaction

The general PCR conditions for reactions performed throughout the study are described (Tables 2.1 and 2.2). Deviations are explicitly indicated, where applicable.

**Table 2.1 Constituents of polymerase chain reactions employed.**

| Reagent | Volume (µl) | Final concentration |
|---|---|---|
| 10× reaction buffer containing 15 mM $Mg_2Cl$ | 5 | 1× |
| Forward primer (10 pmol/µl) | 1 | 0.2 µM |
| Reverse primer (10 pmol/µl) | 1 | 0.2 µM |
| dNTP mix (10 mM each) | 1 | 200 µM of each dNTP |
| $dd_iH_2O$ | variable | - |
| DNA (1 pg-100 ng) | variable | - |
| Thermostable DNA polymerase (*Taq*) | variable | 2.5 - 5.0 U/reaction |
| Final volume | 50 | |

- Where optimisation was required, the final $Mg_2Cl$ concentration was varied from 2.0 to 6.0 mM.
- DNA template varied from 1 pg plasmid DNA to 100 ng genomic DNA.
- If a hot start step was included, the manufacturer's instructions were followed relating to the initial denaturation time period. This varied from 2 minutes to 15 minutes. The use of TaKaRa Ex *Taq* DNA polymerase (Takara Bio Inc.) required a

"cool start" procedure, where tubes were added to the thermocycler block preheated to 94°C.

**Table 2.2 Cycling conditions applied to polymerase chain reactions.**

| | Time | Temperature (°C) | Number of cycles |
|---|---|---|---|
| Hot start | 2 – 15 minutes | 94 or 95 | 1 |
| Initial denaturation | 5 minutes | 94 or 95 | 1 |
| Denaturation | 30 seconds | 94 or 95 | 25-35 |
| Annealing | 30 seconds | 55 | |
| Elongation | 1 minute / kb | 72 | |
| Final elongation | 7 minutes | 72 | 1 |
| Hold | ∞ | 15 | - |

### 2.2.7 Automated DNA sequencing

Automated DNA sequencing was performed on two ABI PRISM 310 Genetic Analyzers (Perkin Elmer), one in the Department of Chemical Pathology, Faculty of Medicine, (former) University of Natal and the other at the South African Sugar Association Experimental Station, Mount Edgecombe. At the CRI/UCI, an ABI PRISM 377 DNA Sequencer (Perkin-Elmer) was used. The author did not operate any of these automated DNA sequencers, but he did prepare the samples to be loaded on the sequencers. For sample preparation, two commercially available kits were initially used, *viz.* the Thermo Sequenase Dye Terminator Cycle Sequencing Premix Kit (Amersham Biosciences) and the

ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Each of the suppliers offered a choice of sample preparation and clean-up procedures, one of each of which was adapted for use in the Molecular Section of the Department of Virology. Unless otherwise stipulated, all dye-termination sequencing reactions were performed in a GeneAmp PCR System 2400 (Perkin Elmer).

### 2.2.7.1 Sample preparation using the Thermo Sequenase kit

Composition of sequencing reaction:

| | |
|---|---|
| Sequencing reagent premix | 8 µl |
| Primer | 5 pmol minimum |
| Plasmid DNA | 200 – 250 ng |
| $dd_iH_2O$ | variable |
| Total volume | 20 µl |

Cycling conditions:

| | | |
|---|---|---|
| 96°C | 1 min | |
| 96°C | 30 sec | × 25 cycles |
| 45°C | 15 sec | |
| 60°C | 4 min | |
| 4°C | ∞ | |

Clean-up:

1. When cycling was complete, each 20 µl sequencing reaction was transferred to a 1.5 ml microcentrifuge tube, and the extent of volumetric loss (owing to evaporation from ineffectively sealed PCR tubes), when it occurred, was noted.
2. A volume of 7 µl of 7.5 M ammonium acetate was added to each tube.
3. The tube contents were brought to a final ethanol concentration of 70% by the addition of 2.5 volumes (68 µl) of cold (–20°C) 100% ethanol, and mixed thoroughly using a vortex mixer.
4. Tubes were centrifuged in a microcentrifuge at maximum speed (13,000 rpm; ~15,000 × *g* using an 18-position rotor) for 15 min. The directional position of tubes was noted.
5. Supernatants were carefully aspirated using a 100 µl pipette, and a different pipette tip for each sample. Residual droplets on the walls of the tubes were absorbed with finely rolled tissue paper.
6. The DNA pellets were washed with 300 µl 70% ethanol and centrifuged for 5 min.
7. The supernatants were removed as above and the pellets were air dried.
8. Each pellet was dissolved in 4 µl of Formamide Loading Dye and vortexed vigorously for 10–20 sec to ensure complete resuspension. The tubes were centrifuged briefly to collect the sample at the bottom of the tubes.
9. Samples were heated to 70°C for 2–5 min to denature, and then placed on ice until being loaded on the automated sequencer.

2.2.7.2 Sample preparation using the FS Terminator and BigDye kits

The same procedures were followed in the application of the FS Terminator and BigDye kits, except where indicated.

Composition of sequencing reaction:

| | |
|---|---|
| Terminator Ready Reaction Mix | 8.0 µl |
| Plasmid DNA | 350 ng |
| Primer | 3.2 pmol |
| $dd_iH_2O$ | variable |
| Total volume | 20 µl |

Samples were mixed well and spun briefly prior to cycle sequencing.

Cycling conditions:

| | | |
|---|---|---|
| 96°C | 10 sec | × 25 cycles |
| 50°C | 5 sec | |
| 60°C | 4 min | |
| 4°C | ∞ | |

Clean-up:

1. The contents of each tube were spun down in a microcentrifuge and transferred into 1.5 ml microcentrifuge tubes, taking note of any volumetric losses.
2. The following were added to each tube, to give a final ethanol concentration of 60 ± 3%:

16 µl of $dd_iH_2O$

64 µl of non-denatured 95% ethanol

3. Tubes were closed, vortexed briefly, and left at room temperature for 15 to 20 minutes to precipitate the extension products. (For FS Terminator kit, the samples were placed on ice for 10 minutes).

4. The tubes were placed in a microcentrifuge and their orientations marked.

5. The tubes were centrifuged for 20 minutes at maximum speed (13,000 rpm; ~15,000 × *g* using an 18-position rotor).

6. Supernatants were carefully aspirated using a 100 µl pipette, and a different pipette tip for each sample. Residual droplets on the walls of the tubes were absorbed with finely rolled tissue paper to ensure complete removal of unincorporated dye terminators present in the supernatants. This step was required to be performed immediately after centrifugation in step 5 finished. When this was not possible (*e.g.* when batches of more than six sequencing reactions were processed concurrently), tubes were centrifuged for a further two minutes, and the supernatants removed immediately thereafter.

7. The pellets were washed by the addition of 250 µl 70% ethanol, followed by brief vortexing and centrifugation for ten minutes at maximum speed, in the same orientation as in steps 4 and 5 above. (For FS Terminator, pellets were merely washed in 250 µl 70% ethanol. Ethanol was carefully aspirated so as not to disturb the pellet. No centrifugation step was included. A Kimwipe was used to remove all residual ethanol traces from the sides of the tubes. The pellets were dried in a vacuum centrifuge).

8. The supernatants were aspirated as in step 6 above.

9. Optionally, the washing step was repeated.

10. The tubes were placed with the lids open in a heat block at 90°C for 1 minute to dry the samples.

11. Each sample pellet was resuspended in 20 µl (later 15 µl) Template Suppression Reagent (TSR).

12. Samples were vortexed and spun down.

13. Samples were heated at 95°C for 2 minutes to denature, and then kept on ice.

### 2.2.8 DNA sequence manipulations

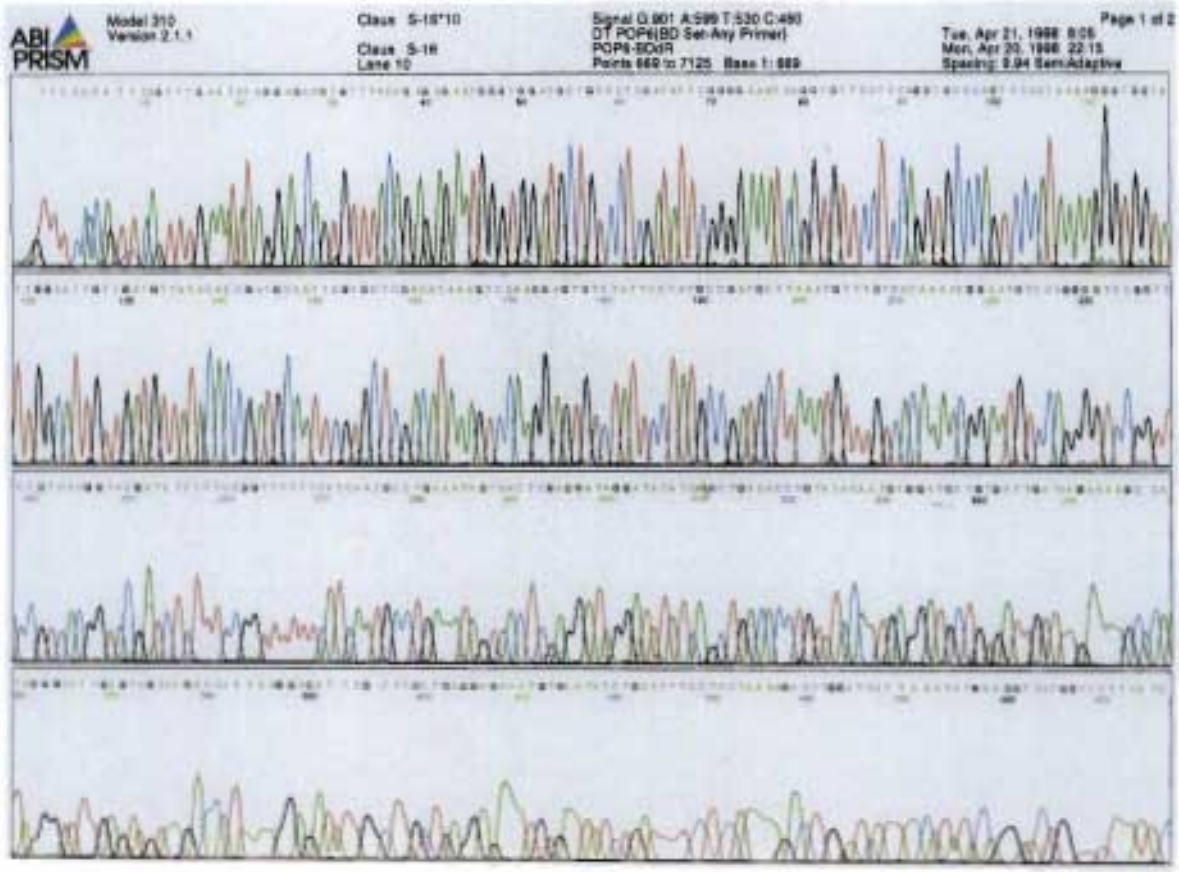



**Figure 2.1 Example of an electrofluorescent profile printout**, as used in this study. The corresponding text file containing the sequence was truncated at position 480, where base calls could still be made with confidence. Each sequence was manually edited base by base.

Sequencing outputs were provided in the form of electrofluorescent profile printouts (Fig. 2.1) and corresponding plain text files of the nucleotide sequences for every sample. Prior to any form of analysis, each printout was manually edited one base at a time to verify, and where applicable, correct the base-calling of the software associated with the sequencers. Any changes were recorded in the corresponding text files, which had been opened in DNAsis for Windows, Version 2.1 (Hitachi Software Engineering Co., Tokyo) (Fig. 2.2).

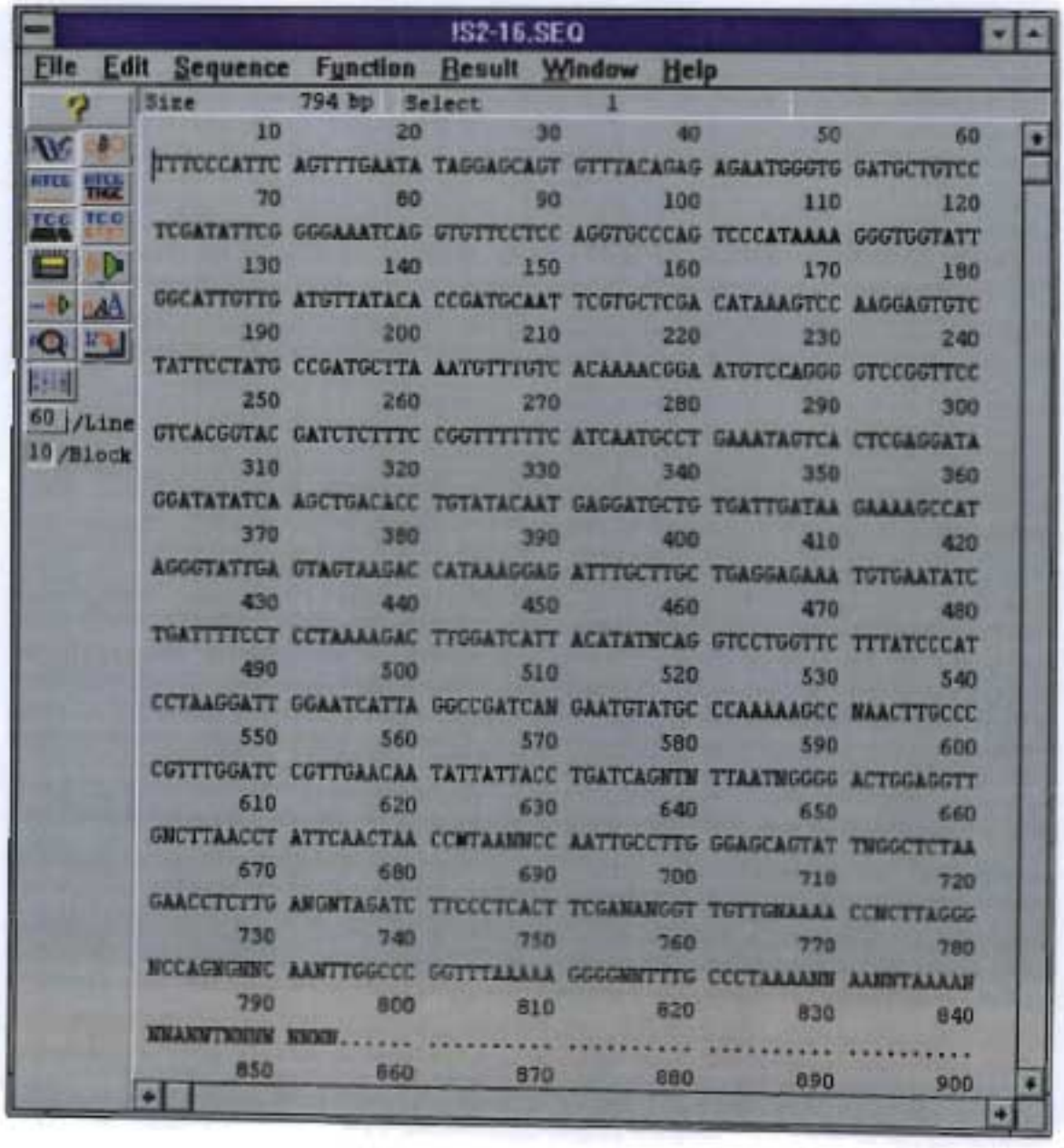



**Figure 2.2 DNAsis sequence illustration in text format.** The same sequence as in Figure 2.1 is shown, prior to truncation or editing. Any residues found to be different from the sequencer software-generated base calling were changed in this type of file.

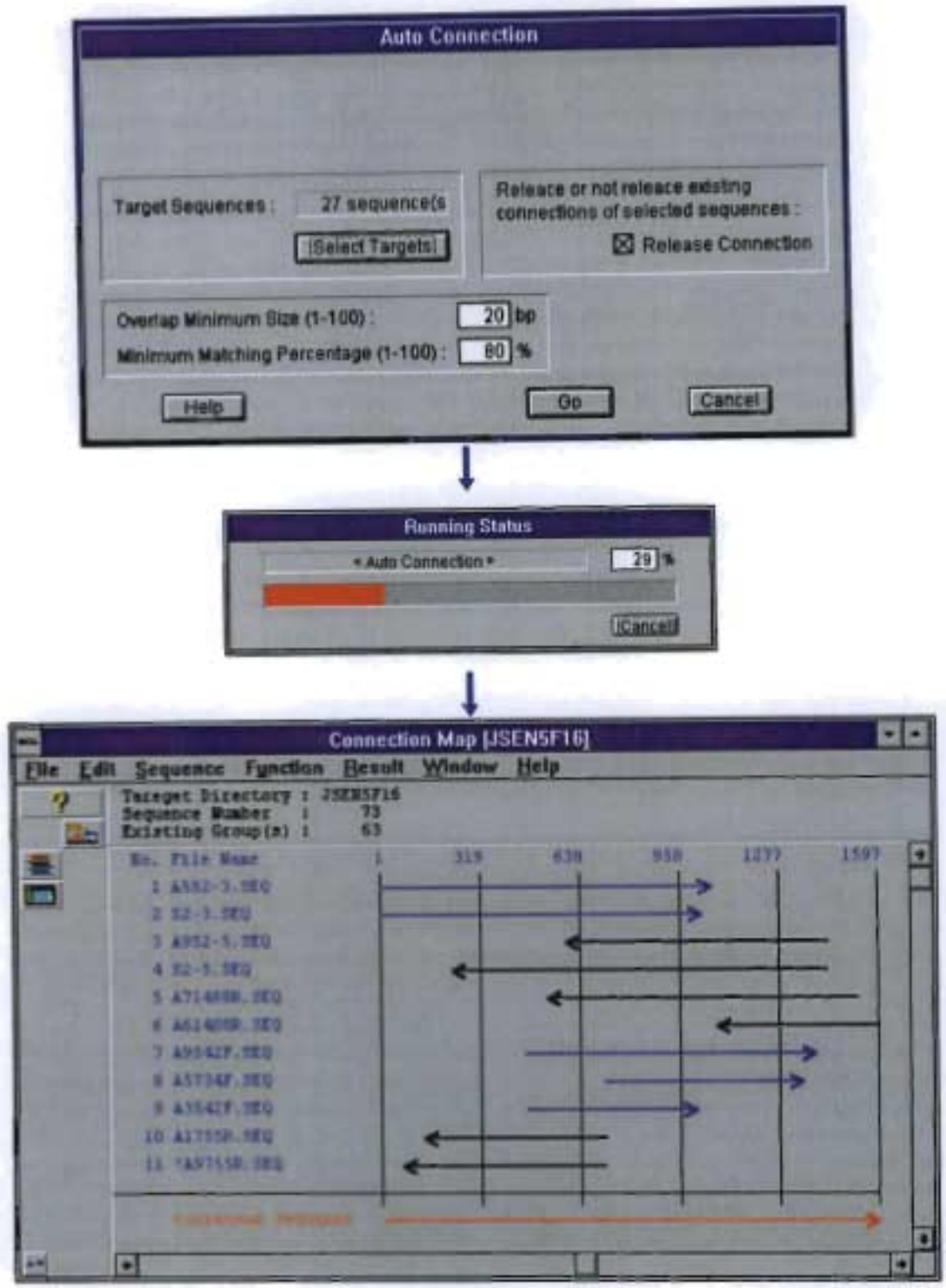



**Figure 2.3 Generation of sequence contigs in DNAsis.** Edited sequences were selected for grouping into contigs (top panel) and the sequence overlap conditions were set. The software read all selected sequences in both directions and, where applicable, assembled them into contigs of semi-overlapping sequences. These could be displayed graphically (bottom panel) such that their directions and relative positions were shown.

The "Contig Manager" function of the DNAsis software was then used to progressively align all edited sequences. It reads each sequence in both directions and aligns any regions of overlap in accordance with the overlap parameters stipulated by the user (Fig 2.3, top panel). Sets of partially overlapping sequences are assembled into groups referred to as "contigs", which the software can then represent in different forms (Fig. 2.3, bottom panel; Fig. 2.4).

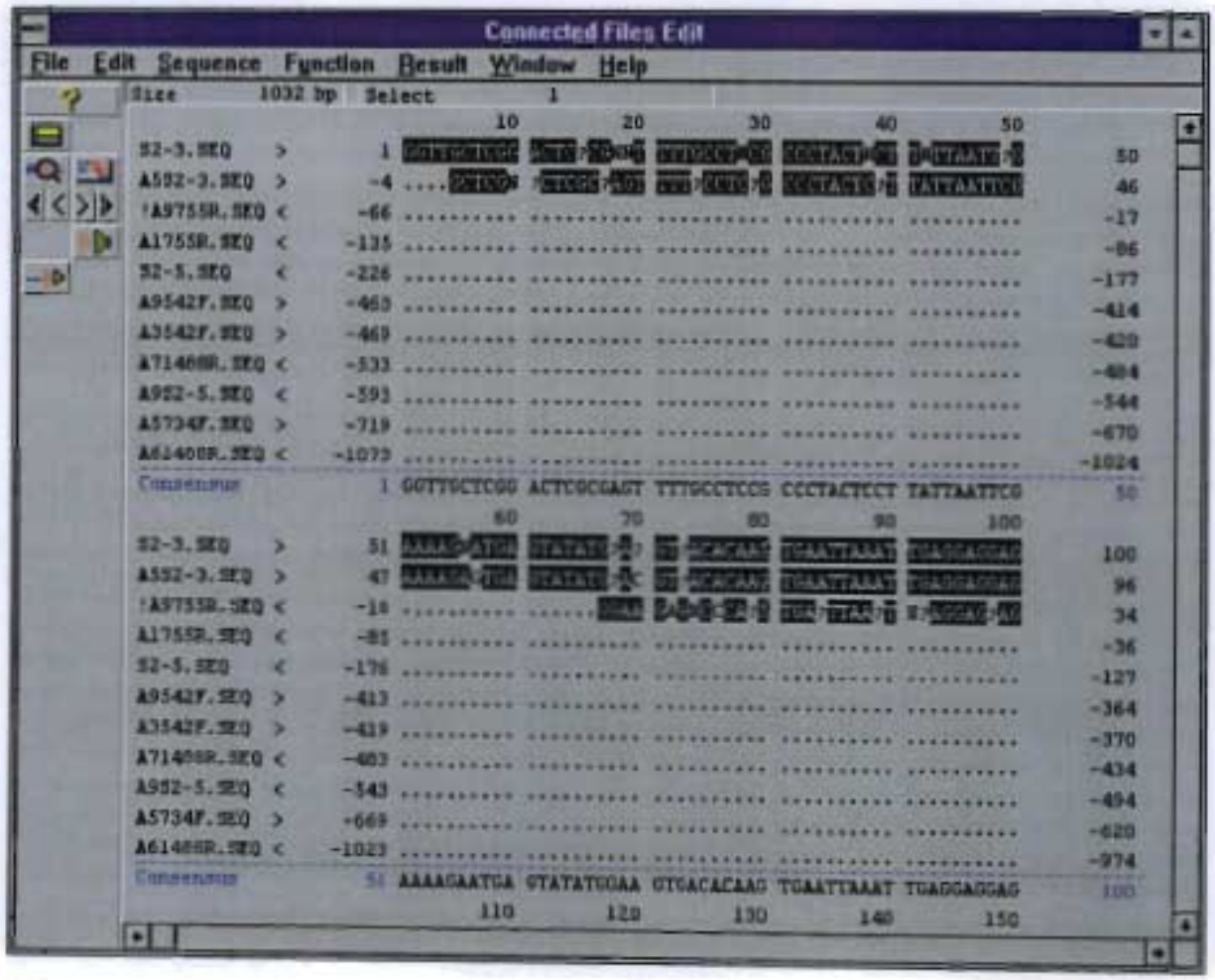



**Figure 2.4 DNAsis alignment view of sequences in a contig.** Each sequence in a contig is displayed in the direction corresponding to the primers used for amplification, and aligned with other sequences in the regions of overlap. Discrepancies were resolved by reference to the electrofluorescent profile printouts.

### 2.2.9 PCR and sequencing primer design

Additional sequencing primers, based on enJSRV sequences obtained, were designed with the aid of OLIGO 4.1 Primer Analysis Software (National Biosciences, Inc.). OLIGO 4.1 was a DOS-based programme. The instructions from the Help menu were followed to optimise primer design.

PCR primers based on conserved JSRV and enJSRV regions identified as part of this study were designed according to the positions of the conserved regions in nucleic acid sequence alignments generated in this study. OLIGO 4.1 was employed to determine the optimal length of the PCR primers such that primer pairs had compatible annealing temperatures. [See Appendix II for sequences of PCR primers designed specifically for this study.]

### 2.2.10 Phylogenetic analyses

Sequences were collected in various versions of BioEdit software (Hall, 1999). The latest versions were used, as they became available. Individual sets of sequences to be aligned were saved in FASTA format and opened in the DOS-based Clustal W (Thompson *et al.*, 1994), and later the Windows-based Clustal X (Thompson *et al.*, 1997) software. The sequences were aligned using this software, and the alignments saved as FASTA files, to be viewed and manually edited in BioEdit. Edited alignments were imported into the latest available versions of GeneDoc (Nicholas & Nicholas, 1997), and trimmed on either end such that all sequence stretches analysed were of the same length. The trimmed alignments were exported in PHYLIP format to be analysed in the latest available versions of the

Phylogeny Inference Package (PHYLIP) (Felsenstein, 1989). The PHYLIP files containing the alignments were opened in the latest versions of Molecular Evolutionary Genetics Analysis (MEGA) software (Kumar *et al.*, 1994; Kumar *et al.*, 2001; Kumar *et al.*, 2004) to obtain the base frequencies and the transition/transversion mutation ratios for each alignment analysed. These are required for later analyses in PHYLIP. Estimates of branch lengths of neighbour-joining trees were calculated using the "Dnadist" and "Neighbor" programmes within PHYLIP. Consensus trees were generated by use of 1,000 bootstrap sampling replications in "Seqboot", followed by analysis using "Dnadist", "Neighbor" and "Consense". The resulting output files were saved with file extensions ".tre", to be opened, viewed and manipulated for illustration and publication purposes in the latest available versions of TreeView (Page, 1996).

## 2.3 RESULTS

### 2.3.1 Sequencing of full-length endogenous JSRV loci

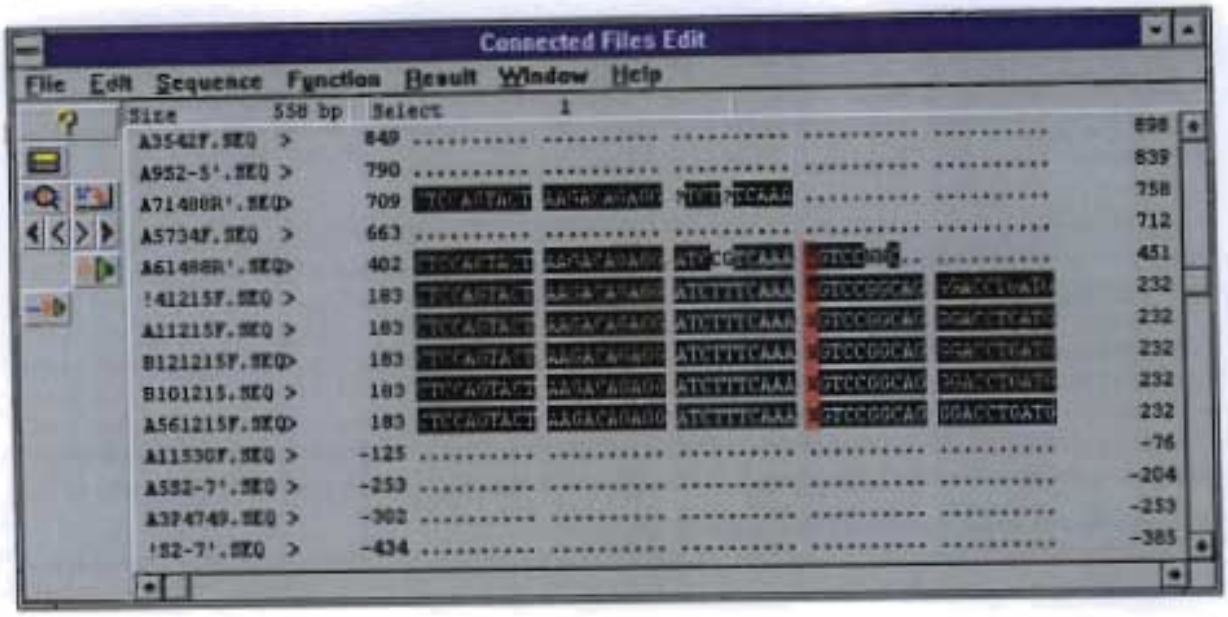
The author was provided with three aliquots of plasmid clones of endogenous JSRV loci, as described in Section 2.2, as well as two sets of JSRV primer aliquots. Sufficient quantities of plasmid DNA of sufficiently high purity for use in sequencing reactions were generated for each of the clones by transformation of DH5α competent cells (Clontech), expansion of transformed clones by culture in LB medium, and plasmid DNA isolation, using a Plasmid Maxi Kit (Qiagen). The primer aliquots were gifts from Dr. Massimo Palmarini and were based on the sequence of JSRV-SA (York *et al.*, 1992). The primers had been synthesised before Dr. Palmarini had embarked on his post-doctoral research at the CRI/UCI, and he was unable at the time to provide the sequences of any of these primers. The names of most of the primers also did not provide information regarding their positions in the JSRV genome. Set 2 was simply numbered 1 through 21. In Set 1, some primers had been assigned names that could subsequently be seen to relate to their positions within the JSRV genome; others related to positions within specific genes; whereas the rest had names that did not relate in any obvious way to any part of the JSRV genome. No clear distinction could be made *a priori* between these three categories within Set 1, however, and the directions (*i.e.* forward or reverse) of many of the primers in Set 1 were also unknown. The directions and positions of primers in Set 2 were all unknown.

Since the two sets of primers essentially constituted random JSRV-specific primers, the author employed a "shot-gun sequencing" approach. This entailed setting up sequencing reactions in batches of six or eight, with each reaction using a different primer. Initially the

FS Terminator kit from PE Applied Biosystems (properly known as "ABI PRISM Dye Terminator Cycle Sequencing Core Kit With AmpliTaq DNA Polymerase, FS") was used to set up sequencing reactions. Within three months, Thermo Sequenase (Amersham Biosciences) as well as BigDye (PE Applied Biosystems) chemistries were introduced into the project (see Fig. 2.5).

Of the 43 primers available, over half yielded positive results in terms of DNA sequence outputs, and the obtained sequence fragments were aligned with the aid of DNAsis for Windows, Version 2.1. This gave rise to a number of overlapping groups of sequences, called contigs, which represented more than 75 % of the endogenous genome. Once all the sequence data that could be obtained from the use of the JSRV-SA-based primers had been generated, eight new sequencing primers were designed (based on the accrued sequence information of enJS5F16) using Oligo 4.1 software. The resulting sequences allowed the merger of the separate contigs, as well as the elucidation of the 5' and 3' LTR sequences. In all, 64 successful sequencing reactions were required to complete the sequence of the first full-length endogenous JSRV locus (Table 2.3), and a further 23 for the 5' and 3' flanking regions. Every part of the genome was covered by at least one forward and one reverse reaction, or by three reactions in the same direction, using at least two different primers. In the event where there was even a single nucleotide discrepancy between a forward and a reverse sequence, or amongst three sequences in the same direction, the sequences were repeated, or new primers were designed in order to address such ambiguities. This was done to ensure that artefacts that can arise from the sequencing reactions were not misinterpreted as true sequence data. One such problem area is shown in Figure 2.5; it was resolved by use of Thermo Sequenase chemistry in place of the FS Terminator kit, and an additional reverse primer.

**A.**




**B.**

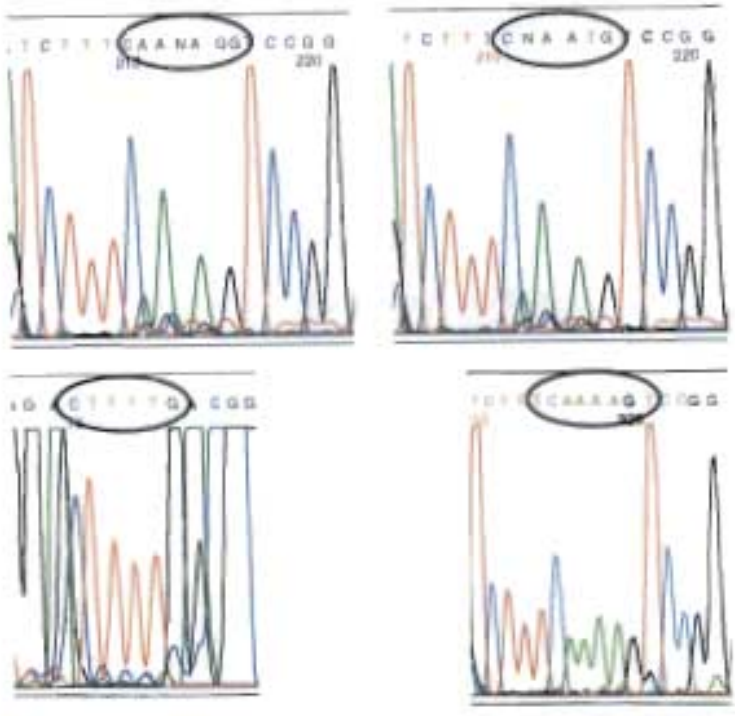


**Figure 2.5 Resolution of sequencing ambiguities by use of alternative sequencing chemistries and design of new primers. A.** A problematic area is highlighted in the DNAsis sequence alignment and editing window. The four sequences displaying the "N" had all been obtained by use of the FS Terminator kit. The sequence shown as "!41215F.SEQ" was generated using Thermo Sequenase, as was the reverse sequence "A61488R'.SEQ". **B.** The two top panels show the ambiguous region, as obtained by use of a forward primer and the FS Terminator kit. The lower panels show the same region, using Thermo Sequenase. The lower left panel represents a reverse sequence of the same area.

Comparison of the enJS5F16 sequence with that of JSRV-SA revealed, amongst numerous minor mutations, that enJS5F16 is marked by two large deletions of 154 and 872 bp in *pol*, the second of which causes a 5' truncation in *orf-x* (Fig. 2.6; Table 2.4). In order to determine if this was a distinguishing feature of endogenous JSRV loci, the *pol* region of enJS59A1 was sequenced (the choice of relevant primers could, by this stage, be determined from the sequence data generated for enJS5F16). It was shown to be of the same length as the *pol* region from JSRV-SA, even though the open reading frame was interrupted by a stop codon. However, it did represent an uninterrupted open reading frame in *orf-x*.

**Table 2.3** **Number of sequencing reactions required and number of primers used to elucidate the complete nucleotide sequence of clones enJS5F16, enJS59A1 and enJS56A1.**

| Clone | Total number of reactions | Number of primers used, based on sequence of: | | | |
|---|---|---|---|---|---|
| | | JSRV-SA | enJS5F16 | enJS59A1 | enJS56A1 |
| enJS5F16 | 64 | 27 | 8 | - | - |
| enJS59A1 | 64 | 23 | 3 | 4 | - |
| enJS56A1 | 52 | 26 | 4 | 4 | 5 |

The sequencing of enJS59A1 continued, followed by enJS56A1, employing a far more structured strategy than the "shot-gun sequencing" approach used for enJS5F16. This was facilitated by the knowledge that had been gained of the various primer positions. The sequencing of enJS59A1 and enJS56A1 also necessitated the design of new primers (Table 2.3), either because stocks of the JSRV-SA-specific primer aliquots had been depleted, or

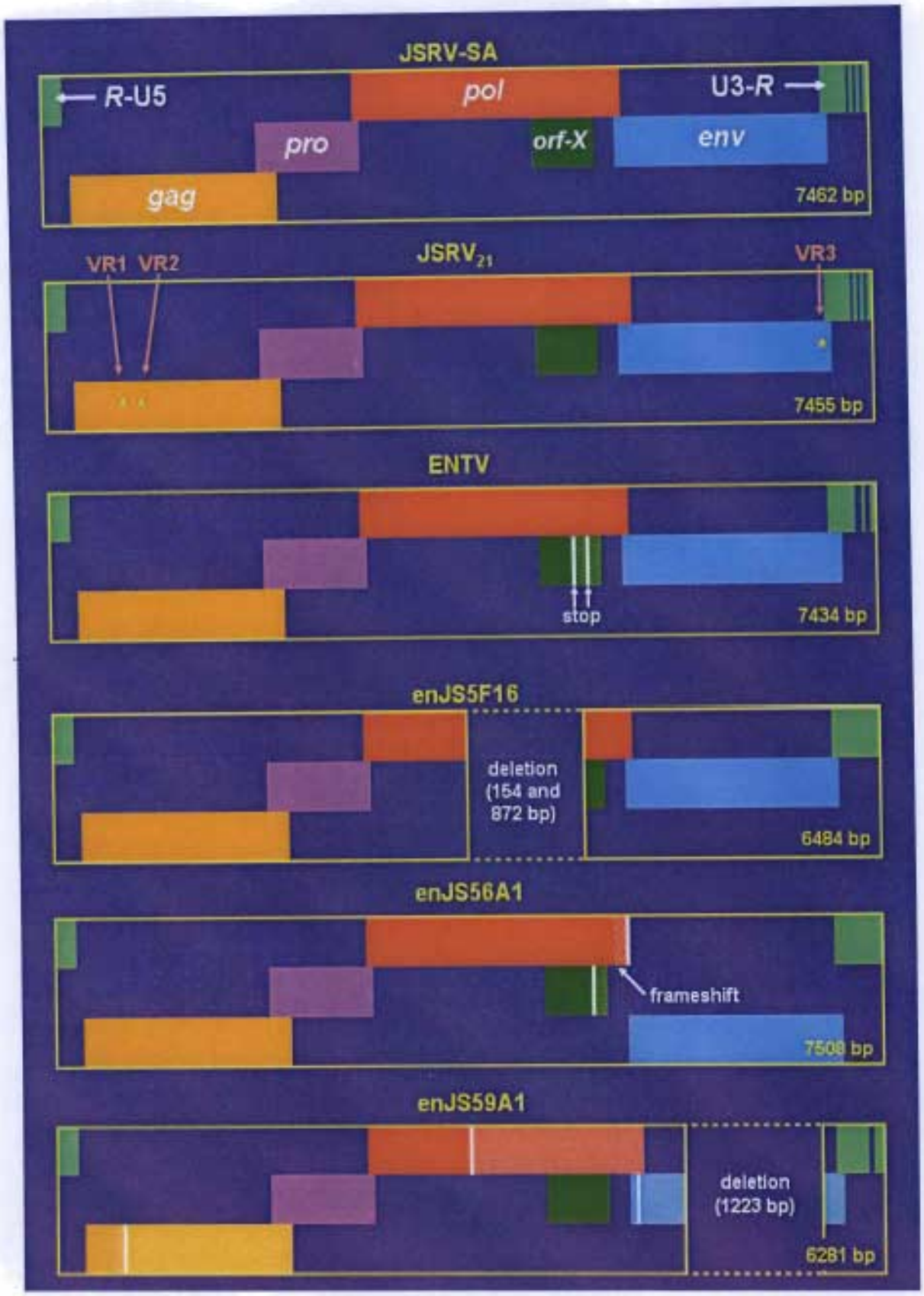



**Figure 2.6 The genomic structure of exogenous and endogenous JSRV-related retroviruses of sheep.** The *gag* open reading frame has been fixed in the same reading frame for all sequences shown. Premature stop codons are indicated by vertical white bars. Sequences after premature stop codons are indicated by diagonal lines. Stretches of different peptide translation resulting from

frameshift mutations are indicated by white dotting. The positions of variable regions of JSRV sequences are indicated in JSRV21 by asterisks. The nucleotide lengths (from the 5' *R* to the 3' *R*) of the sequences are indicated at the bottom right.

because the existing primers did not yield satisfactory results in terms of sequence quality, presumably owing to mismatches with the endogenous sequences.

### 2.3.2 Sequence analysis of full-length endogenous JSRV loci

The sequences of the three full-length endogenous JSRV loci were scanned and analysed, both visually and computationally. The full proviral lengths of enJS5F16, enJS56A1 and enJS59A1 are 6916, 7940 and 6696 bp, respectively. In Table 2.4, the lengths from *R* to *R*, which would delineate the repeated 5'- and 3'-termini of exogenous retroviral RNA genomes (Fig. 1.1), are indicated for ease of comparison with JSRVs. ENTV was included in the comparisons, owing to its very close relationship with JSRV, both in terms of nucleotide sequence and pathogenic function (Cousens *et al.*, 1999). Several noteworthy findings are described in the sections below.

#### 2.3.2.1 Insertion sites and flanking regions

The question of whether or not enJSRVs share common integration sites was addressed by sequencing outwards of the endogenous JSRV genomes beyond the LTRs. The sheep genomic sequences at which each of the insertions occurred, were determined (Fig. 2.7), and it was noted that they were different for all three loci. The lack of homology between

the integration sites indicates that these viruses were most probably inserted randomly into the genomic DNA of their host cells at the time they became fixed in the sheep genome.

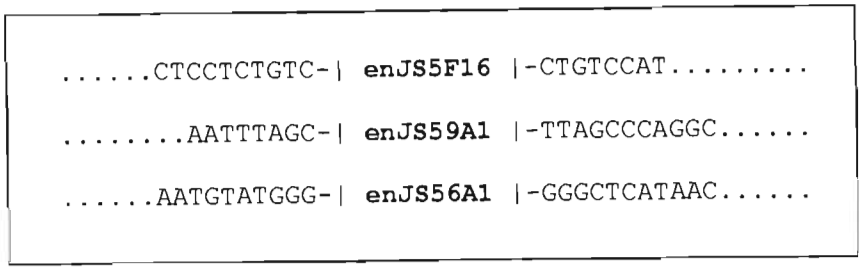



**Figure 2.7 The sites at which each of the three endogenous loci became fixed in the sheep genome by integration are different.**

The entire 5' and 3' flanking regions of penJS5F16, and parts of the flanking regions of penJS59A1 were sequenced. These flanking regions had arisen from the cloning strategy used to isolate the endogenous loci, *viz.* RE digestion of sheep genomic DNA with *Xba*I. The flanking regions therefore constituted those parts of the sheep genome upstream and downstream of the endogenous proviral loci, up to an *Xba*I site. Sequencing of these regions was performed by determining the DNA sequence just outside the loci and designing primers based on those (outward sequencing). Concurrently, primers based on the cloning vector were used to sequence "into" the cloned region from each end. In both instances, new primers were progressively designed and utilised, until the entire flanking region included in penJS5F16 had been assembled. The sequence of the flanking region was interrogated by means of a BLAST search, but no meaningful homology could be identified. However, some information on the vector used for cloning could be elucidated. [It was not clear which version of pBluescript (Stratagene) had been used during the cloning procedure.] The six possibilities could be narrowed down to only two: pBluescript II SK(+) or pBluescript II SK(–).

### 2.3.2.2 Long terminal repeats

All three endogenous proviral loci had an upstream and a downstream LTR, the hallmark of complete proviruses. The U5 regions were highly conserved among all sequences, with the exception of two single base pair deletions in JSRV-SA and ENTV, and a single base pair insertion in enJS59A1. The 13-bp repeat regions (R) of all three clones were identical to those of exogenous JSRVs. The U3 regions of enJS5F16 and enJS56A1 are 98 % homologous to one another, and 91 % homologous to the U3 region of enJS59A1 over 89 % of its length. However, the latter region is marked by a stretch of numerous deletions and mutations relative to the other two endogenous U3 regions, thereby lowering its overall homology to these substantially. The reported finding by (Palmarini *et al.*, 1996a) and (Bai *et al.*, 1996) that exogenous U3 regions are 47 bp shorter than endogenous ones could be confirmed when comparing $JSRV_{21}$ and JSRV-SA with enJS5F16 and enJS56A1. However, no distinct insertions of 30 and 16 bp in the endogenous U3 regions, relative to the exogenous ones, could be identified. No combination of theoretical nucleotide omissions in the enJSRV U3 regions will lead to homology with the corresponding sequences in JSRVs. At least, the homology that can be achieved this way is far lower than that which is observed between regions in the U3 that are naturally conserved amongst the JSRVs. Rather, there appear to be regions of homology, interspersed with regions that lack homology, and the total lengths of the non-homologous stretches of the endogenous sequences are longer, thereby accounting for the 47-bp difference.

An interesting observation regarding the endogenous LTRs is that, whereas the 5' and 3' LTRs of enJS5F16 are identical to one another, those of enJS56A1 and enJS59A1 display two and four base pair changes, respectively. Since retroviral LTRs arise from short

**Table 2.4** **Genomic sequence and feature comparisons of the endogenous JSRV proviral loci enJS5F16, enJS56A1 and enJS59A1 with JSRVs and ENTV, indicating regions homologous to exogenous coding regions.**

| Virus | *R* to *R* / proviral length (bp) | U5 (bp) | Positions and lengths of coding regions, and lengths of deduced polypeptide sequences[1] | | | | | U3 (bp) |
|---|---|---|---|---|---|---|---|---|
| | | | *gag* | *pro* | *pol* | *"orf-x"* | *env* | |
| JSRV-SA | 7462 | 113 | 263 - 2101<br>[1839 bp / 612 AA] | 1993 - 2862<br>[870 bp / 289 AA] | 3108 - 5441<br>[2334 bp / 777 AA] | 4606 - 5106<br>[501 bp / 166 AA] | 5350 - 7197<br>[1848 bp / 615 AA] | 272 |
| $JSRV_{21}$ | 7455 | 115 | 264 - 2099<br>[1836 bp / 611 AA] | 1991 - 2860<br>[870 bp / 289 AA] | 3106 - 5451<br>[2346 bp / 781 AA]<br>stop codon 12 bp downstream relative to JSRV-SA owing to point mutation | 4604 - 5104<br>[501 bp / 166 AA] | 5348 - 7195<br>[1848 bp / 615 AA] | 272 |
| ENTV | 7434 | 113 | 256 - 2097<br>[1842 bp / 613 AA] | 1989 - 2858<br>[870 bp / 289 AA] | 3104 - 5437<br>[2334 bp / 777 AA] | 4602 - 5102<br>[501 bp]<br>two stop codons ∴ no ORF | 5346 - 7199<br>[1854 bp / 617 AA]<br>termination point further downstream compared with JSRV sequences ∴ longer ORF | 250 |
| enJS5F16 | 6484 / 6916 | 115 | 264 - 2114<br>[1851 bp / 616 AA]<br>Two regions in MA[2] lacking homology with JSRVs and ENTV | 2006 - 2875<br>[870 bp / 289 AA] | 3121 - 4428<br>[1308 bp / 435 AA]<br>two large in-frame deletions, of 154 & 872 bp | 3911 - 4093<br>[183 bp]<br>5'-truncation owing to deletion in *pol* | 4337 - 6172<br>[1836 bp / 611 AA]<br>200 bp at 3' end of TM[3] have very low homology to JSRVs and ENTV | 319 |
| enJS56A1 | 7508 / 7940 | 115 | 264 - 2114<br>[1851 bp / 616 AA]<br>Two regions in MA[2] lacking homology with JSRVs and ENTV | 2006 - 2875<br>[870 bp / 289 AA] | 3121 - 5464<br>[2344 bp]<br>deletion of 'AT' at 5322, leading to frameshift; stop codons at 5419 & 5440; final (theoretical) stop codon 12 bp downstream relative to JSRV-SA owing to point mutation | 4619 - 5119<br>[501 bp]<br>stop codon at 5081 owing to point mutation | 5361 - 7196<br>[1836 bp / 611 AA]<br>200 bp at 3' end of TM[3] have very low homology to JSRVs and ENTV | 319 |
| enJS59A1 | 6281 / 6696 | 116 | 262 - 2113<br>[1852 bp]<br>single bp insertion at position 482, leading to frameshift mutation; first stop codon at position 520; two regions in MA[2] lacking homology with JSRVs and ENTV | 2005 - 2874<br>[870 bp / 289 AA] | 3120 - 5465<br>[2346 bp]<br>point mutation leading to TGA stop codon in position 3770; final (theoretical) stop codon 12 bp downstream relative to JSRV-SA owing to point mutation | 4618 - 5118<br>[501 bp / 166 AA] | 5362 - 5986<br>[625 bp]<br>stop codon in position 5398 owing to point mutation; large deletion of 1223 bp thereafter; 200 bp at 3' end of TM[3] have very low homology to JSRVs and ENTV | 301 |

(1) The predicted polypeptide lengths of coding regions interrupted by stop codons are not indicated. (2) MA = matrix region of *gag* (3) TM = transmembrane region of *env*

sequence duplications at the 5' and 3' ends of the genome during reverse transcription, they are always identical at the time of integration. Differences between the 5' and 3' LTRs of endogenous retroviruses are therefore indicative of the time that has passed since their insertions (Mager & Freeman, 1995), and it can be deduced that enJS5F16 represents the most recent integration into the sheep genome, whereas enJS59A1 represents the most ancient one, out of the three loci studied here.

#### 2.3.2.3 The 5' untranslated region (5' UTR)

The $tRNA^{Lys}_{1,2}$ primer binding site immediately downstream of U5 is conserved for all the JSRV sequences, as well as ENTV, indicating that all of these viruses utilise the same class of tRNA molecule to prime second strand synthesis during reverse transcription. The 35-bp region immediately upstream of the *gag* ATG start codon was found to be identical in all JSRV sequences, as well as ENTV (Fig. 2.8). (ENTV-2 has two single nucleotide differences in this stretch compared to ENTV and JSRVs.) This represents the longest sequence of absolute nucleotide sequence homology in a non-coding region of known JSRV sequences, an observation that had not previously been reported. Although the significance of this highly conserved region was not investigated, given its position immediately upstream of the *gag* ATG start codon, it probably relates to the translation of the Gag and Gag-Pro-Pol polyproteins by the host cell translation machinery. Like cellular mRNAs, the translation of JSRV mRNA would necessitate the recruitment of the small and large ribosomal subunits, via the cellular eukaryotic translation initiation factors, to the start codon. This might be achieved via an alternative mechanism to the canonical translation initiation of cellular mRNAs, which would most likely impose specific mRNA

secondary structure constraints on this region, thus accounting for its high degree of sequence conservation.

### 2.3.2.4 The *gag* gene

The *gag* regions of enJS5F16 and enJS56A1 represent full open reading frames with an amino acid sequence identity of the entire predicted polyprotein of 98.2 %, similar to a

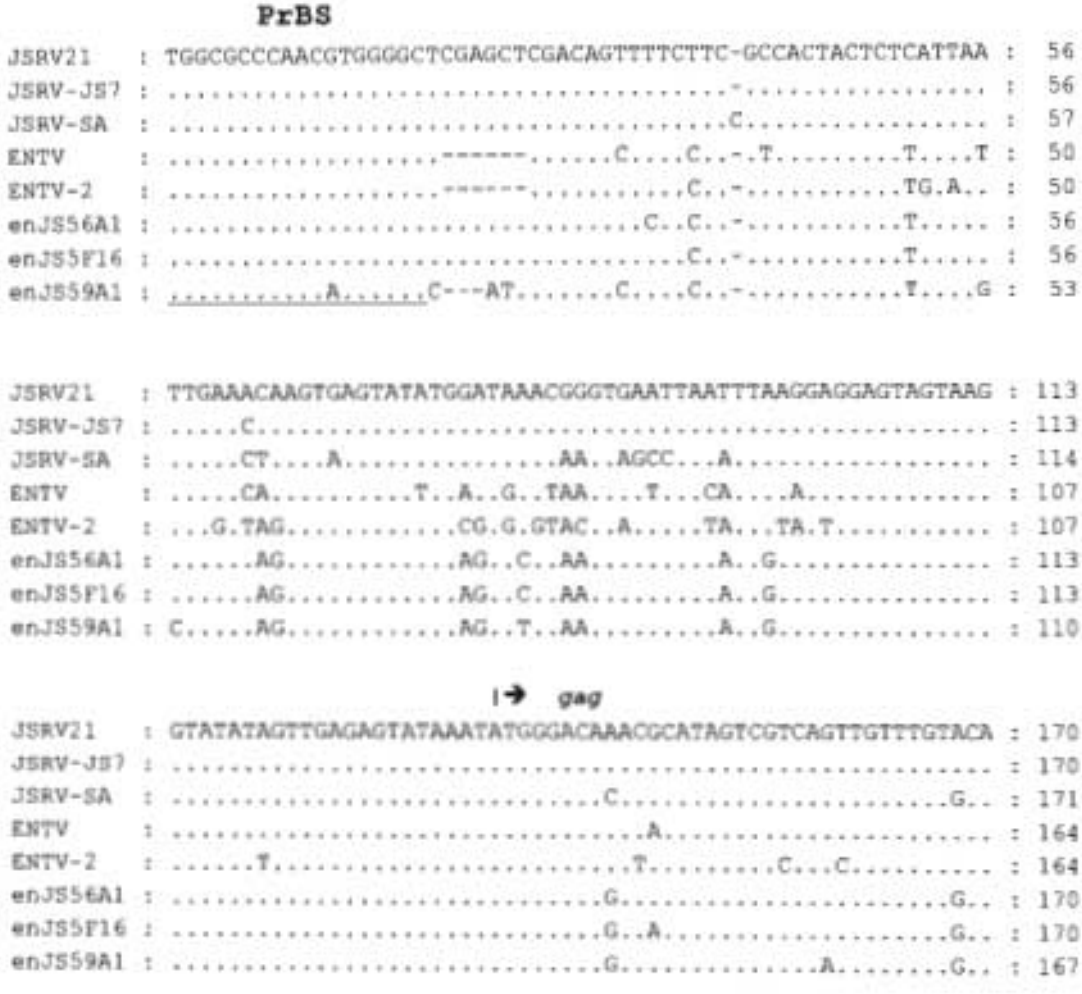



**Figure 2.8 Nucleotide sequence alignment of the 5' untranslated regions of sheep betaretroviruses:** exogenous $JSRV_{21}$, JSRV-SA, and ENTV and the endogenous enJS5F16, enJS59A1, and enJS56A1 proviruses. The primer binding site (PrBS) is underlined and the beginning of the *gag* open reading frame is indicated by an arrow above the ATG start codon. Dots refer to sequences identical to the top sequence, while dashes indicate lack of sequence.

comparison of the same regions in JSRV-SA and $JSRV_{21}$. When comparing these endogenous and exogenous sequences, their predicted AA homology is around 93 %. The discrepancy is attributable almost entirely to two regions of 75 and 117 base pairs between the predicted matrix (MA) and capsid (CA) parts of *gag* [*i.e.* in p15 (Murcia *et al.*, 2007)] that are conserved in all three endogenous loci. These regions yield no meaningful amino acid identity when compared to their equivalent regions in JSRVs and ENTV. Notably, the

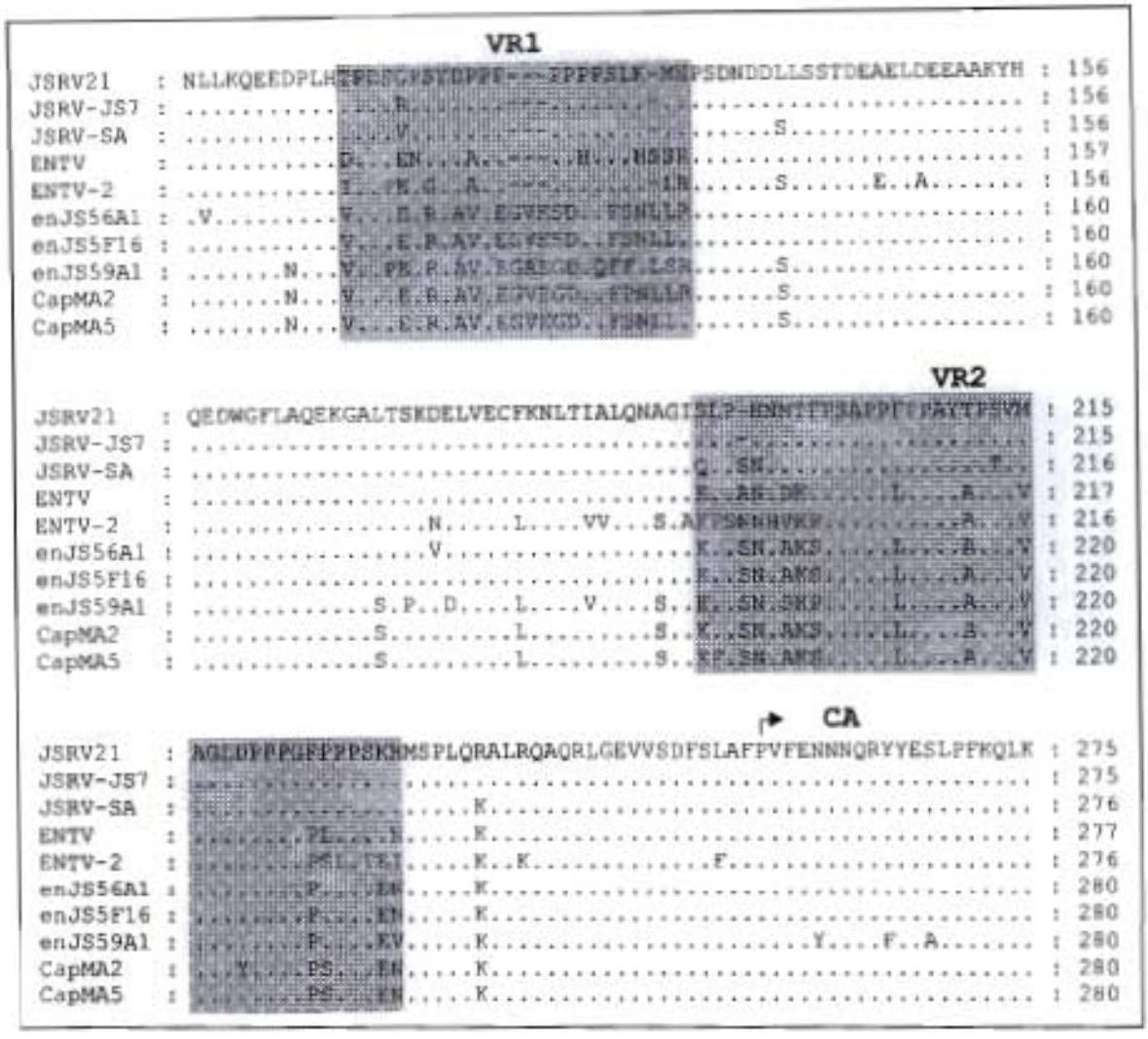



**Figure 2.9 Alignment of a selection of the deduced *gag* amino acid sequences of sheep betaretroviruses.** The single base pair insertion in enJS59A1 *gag* was corrected to create its deduced amino acid sequence and facilitate its inclusion in this alignment. VR1 and VR2 are indicated by shaded areas. Note that the proline residues in VR1 of the exogenous sequences are absent in the endogenous proviruses. The beginning of the putative CA region is indicated. Endogenous sequences CapMA2 and CapMA5 were obtained by PCR amplification from goat DNA.

75-bp region also exhibits polymorphism between JSRV and ENTV in that there is only 50% identity at the amino acid level compared to the 95.8% identity for the entire Gag polyprotein. This region was termed VR1 (variable region 1) for the betaretroviruses of sheep (Fig. 2.9). VR1 is 12 bp shorter in JSRVs than in enJSRVs, and it encodes a stretch of seven consecutive proline residues in exogenous JSRVs, five of which are conserved in ENTV. This proline-rich region is not encoded by any of the three enJSRVs. This finding had not been reported previously and appears to represent a highly conserved difference between endogenous and exogenous JSRV sequences, at both the nucleotide and the amino acid level.

Approximately 150 base pairs downstream of VR1 is another region of polymorphism between endogenous and exogenous JSRV sequences, which was termed VR2. It is also relatively proline rich for both endogenous and exogenous viruses. It is interesting that ENTV shows a closer relationship to the endogenous loci in VR2 than to the exogenous JSRVs.

The *gag* gene of enJS59A1 is marked by a frameshift mutation with a termination codon brought about by a single base pair insertion, and thus does not encode a full open reading frame. Even when correcting for this frameshift, its predicted amino sequence is less similar to the other two enJSRV loci (less than 93 %) than they are to one another. It is also less similar to the exogenous JSRVs than the other two loci, confirming that it seems to represent a more ancient insertion into the sheep genome.

### 2.3.2.5 The *pro* gene

The *pro* gene is the only one that represents an uninterrupted open reading frame of the same length in endogenous and exogenous JSRVs and ENTV. All comparisons yield amino acid sequence identities ranging from 95.2 % to 99.7 %. In their 5' halves, all JSRV-related sequences examined encode the five conserved motifs found in all dUTP pyrophosphatase (dUTPase) enzymes (Fig. 2.10), as already noted by York *et al.* (1992)

| | Motif | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| *E. coli* | AGLDL....RSGLGHK....GLIDSDYQG....GERIAQMI....RGEGGFGHSG | | | | |
| human | AGYDL....RSGLAAK....GVIDEDYRG....GDRIAQLI....RGSGGFGSTG | | | | |
| tomato | AGYDL....RSGWALK....GVIDADYRG....GDRIAQLI....RGSGGFGSTG | | | | |
| yeast | AGYDI....RSGLAVK....GVVDRDYTG....GDRVAQLI....RGAGGFGSTG | | | | |
| HSV-1 | AGYDI....RSSLNAR GLIDSGYRG GAKVAQLV....RGTRGFGSTG | | | | |
| MPMV | AGLDL....RSSITMK....GVIDNDYTG....GNRIAQLI....RGQGSFGSSD | | | | |
| MMTV | AGLDL....RSSNYKK....GVIDSDFQG....GERIAQLL....RGSEGFGSTS | | | | |
| HERV-K10 | AAVDL....RSSLNLK....SVVDSDYKG....RDRIAQLL....KRIGGLVSTD | | | | |
| HERV-K cons. | AAVDL....RSSLNLK....GVVDSDYKG....GDRIAQLL....KRIGGFGSTD | | | | |
| ENTV | AGLDL....RSSASLK....GVIDSDYTG....GQRIAQLL....RQDKGFGSSD | | | | |
| JSRV-SA | AGLDL....RSSASLK....GVIDSDYTG....GQRIAQLL....RQDKGFGSSD | | | | |
| enJS5F16 | AGLDL....RSSASLK....GVIDSDYTG....GQRIAQLL....RQDKGFGSSD | | | | |
| enJS56A1 | AGLDL....RSSASLK....GVIDSDYTG....GQRIAQLL....RQDKGFGSSD | | | | |
| enJS59A1 | AGLDL....RSSASLK....GVIDSDYTG....GQRIAQLL....RQDKGFGSSD | | | | |

**Figure 2.10 Amino acid motifs conserved amongst all known dUTPase enzymes.** JSRV-like viruses have identical motifs. Bold underlined letters indicate residues unique to JSRV-like sequences. HERV-K cons., consensus sequence of HERV-K dUTPases (Harris *et al.*, 1997); HSV-1, herpes simplex virus 1. Table modified from (Harris *et al.*, 1997) (1997) by addition of MPMV, HERV-K consensus and JSRV-related sequences.

pertaining to JSRV-SA. Even though there are numerous differences in the nucleotide sequences encoding these motifs, the mutations are silent and do not manifest at the amino acid level. This stringent conservation may indicate a functional enzyme, even in the endogenous sequences. Three of the five motifs show residues which are unique to the JSRV-like sequences relative to the other sequences used for the comparison in Figure

2.10. This may have implications on the functionality of the encoded enzyme, but a larger number of dUTPases would be required for comparison to establish if these residues are indeed unique to JSRV.

### 2.3.2.6 The *pol* gene

The *pol* gene is marked by major defects in enJS5F16 and enJS59A1: in enJS5F16 there are two large in-frame deletions of 154 and 872 bp, while a point mutation in enJS59A1 creates a stop codon (position 4071 of the provirus sequence) in the reverse transcriptase domain (Fig. 2.6; Table 2.4). Translation of an enJS59A1 transcript (assuming that this locus is transcribed) would thus be aborted after having processed only 28 % of *pol*. enJS56A1 harbours a 2-bp deletion with respect to exogenous JSRVs at the 3' end of the *pol* gene (corresponding to the end of the integrase [IN] domain) that would yield a polypeptide 14 amino acids shorter compared to the native JSRV IN, with the last 33 amino acids having no similarity due to the frameshift. At the nucleotide level of *pol*, homology between JSRV-SA and the two more recently integrated loci is in excess of 98% (disregarding the deletions in enJS5F16). Homology with enJS59A1 is 93%. By comparison, the homology between JSRV-SA and $JSRV_{21}$ *pol* genes is 96.3%. This coding region, which includes *orf-X*, is thus highly conserved at the nucleotide level.

Another observation relates to the 3' termination signals of *pol* translation (albeit theoretical in the case of the three endogenous loci) in the JSRV-related sequences (Fig. 2.11). It can be seen that ENTV, ENTV-2, JSRV-SA and enJS5F16 share the same termination signal, whereas the *pol* regions of JSRV21, JSRV-JS7, enJS56A1 and

enJS59A1 end four codons further downstream. All but the ENTV sequence share the downstream stop codon, even though it would not be used in those sequences that are already terminated earlier.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ENTV | GAC | ATT | GAA | **TGA** | GAC | CAC | GAA | TGG | ACT | ACC |
| ENTV-2 | GAC | ACT | GAG | **TGA** | GCC | TAC | GAG | **TGA** | GCT | ACC |
| JSRV-SA | GAC | CCT | GAG | **TGA** | GCC | CAC | GAG | **TGA** | GCT | GCC |
| JSRV21 | GAC | GCT | GAG | CGA | GCC | CAC | GAG | **TGA** | GCT | GCC |
| JSRV-JS7 | GAC | GCT | GAG | CGA | GCC | CAC | GAG | **TGA** | GCT | GCC |
| enJS5F16 | GAC | ACT | GAG | **TGA** | GCC | CAC | GAG | **TGA** | GCT | GCC |
| enJS56A1 | GAC | ACT | GAG | CGA | GCC | CAC | AAG | **TGA** | GCT | GCC |
| enJS59A1 | GAC | ACT | GAG | CGA | GCC | CAC | GAG | **TGA** | GCT | GCC |

**Figure 2.11 Termination sequences of *pol* in sheep betaretroviruses.** Stop codons are marked in bold letters.

### 2.3.2.7 Open reading frame "x"

The *orf-x* region (the alternate reading frame in *pol*) is uninterrupted in enJS59A1. In enJS5F16, *orf-x* is marked by a major truncation as a consequence of the deletion in *pol* (Fig 2.6), while in enJS56A1 there is a stop codon 39 bp before the usual stop codon in exogenous JSRV *orf-x*.

### 2.3.2.8 The *env* gene

The majority of *env* (1223 bp of 1836 bp) is deleted in enJS59A1 (Fig 2.6). In addition, it is marked by an early stop codon only 36 bp downstream of the AUG start codon. In enJS5F16 and enJS56A1, however, *env* is a full open reading frame with 98% amino acid identity when comparing the two sequences, and ~92% identity when comparing either of

them to JSRV-SA or $JSRV_{21}$ *env*. The last 67 amino acids of *env* (in the transmembrane domain) represent another region of high divergence (only ~58% amino acid identity) between exogenous and endogenous JSRV sequences (Fig. 2.12). A significant degree of

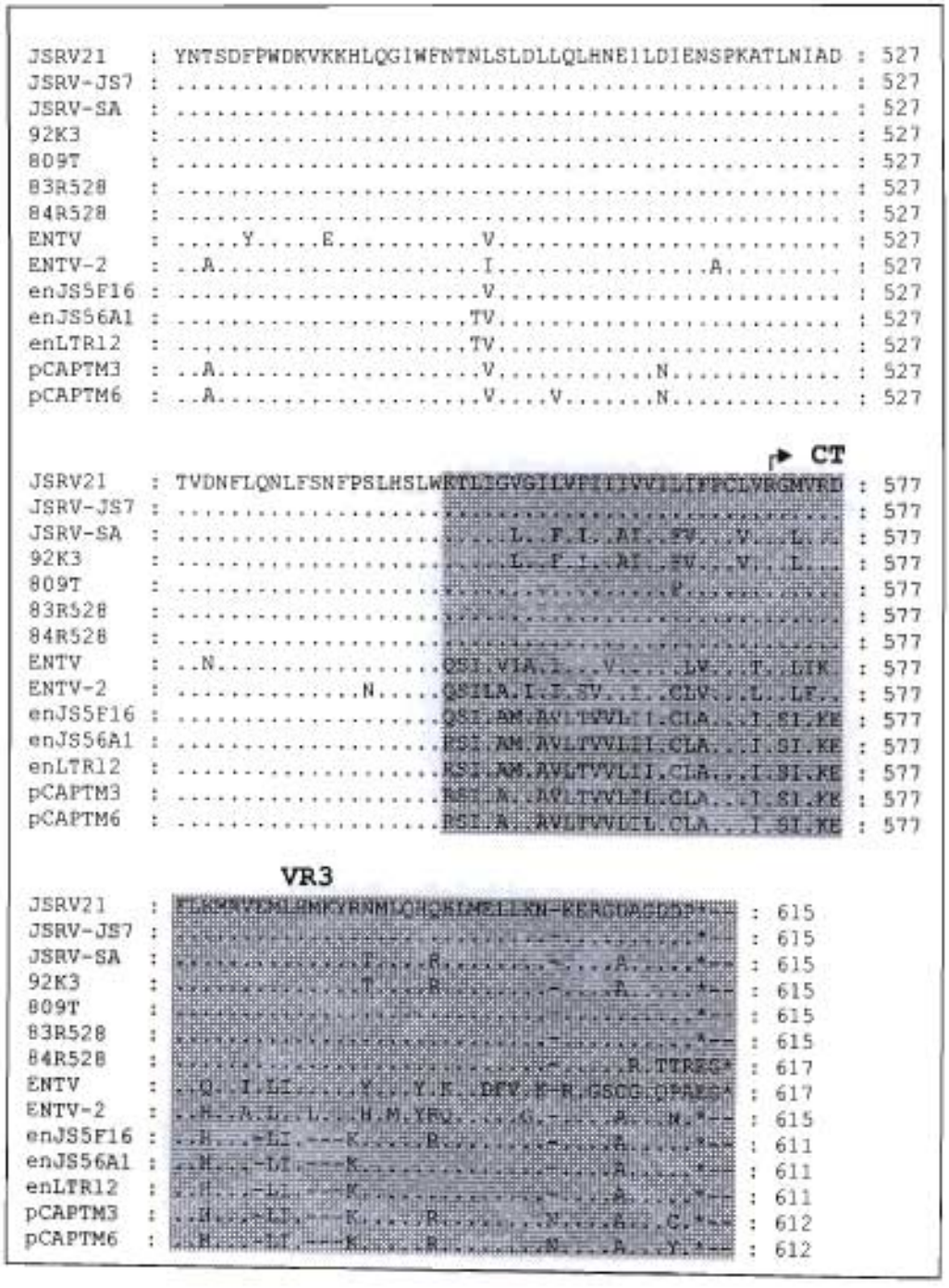



**Figure 2.12 Alignment of deduced *env* amino acid sequences in the transmembrane region of sheep betaretroviruses and JSRV-related endogenous sequences.** VR3 is highlighted by shading. The beginning of the cytoplasmic tail domain is indicated.

variability exists between exogenous JSRV and ENTV sequences in this area as well. [See Appendix I for GenBank accession numbers relevant to this thesis.] It is even highly variable between JSRV type I (African) and JSRV type II (UK and USA) isolates compared to other parts of the genome, and was termed variable region 3 (VR3) of sheep betaretroviruses.

### 2.3.2.9 Restriction profile analysis of the three endogenous JSRV loci

An attempt was made to classify the three full-length enJSRV sequences according to restriction profiles, analysing them with the same eleven restriction endonuclease recognition sites used by Bai *et al.*(1996) in their classification of enJSRVs into six different loci (*cf.* Section 1.4, and Figure 1.2 therein):

enJS56A1: The restriction profile of this sequence corresponds to locus 1, including the distinguishing *Eco*RI site of the Group I sequences, with the exception of a *Pst*I site which it shares with JSRV-SA. None of the loci described by Bai *et al.* (1996) share this site with JSRV-SA.

enJS5F16: This sequence has the same restriction profile as enJS56A1 (including the JSRV-SA-derived *Pst*I site). In addition, it has another *Pst*I site which, according to Bai *et al.* (1996), was the distinguishing feature of locus 4 enJSRVs. This sequence, however, also exhibits the *Eco*RI site purportedly characteristic only of Group I sequences.

enJS59A1: This sequence lacks the *Eco*RI site that identifies Group I loci. It is missing four different recognition sites that are conserved amongst all JSRV sequences in the system used by Bai *et al.* (1996), both endogenous and exogenous. These missing sites

are not attributable to deletions in this clone, but to point mutations. In addition, this sequence has a *Pst*I site and an *Xho*I site that are not included in any of the sequences used by Bai *et al.* (1996) in the establishment of their classification system.

Owing to the fact that some of the above findings, relating to the three endogenous clones, were not in keeping with the classification system of Bai *et al.* (1996), the exogenous $JSRV_{21}$ sequence (Palmarini *et al.*, 1999a) was analysed according to the same criteria:

$JSRV_{21}$: This sequence is missing four different recognition sites, the presence of which is expected to identify exogenous JSRVs. It is missing five sites that are meant to be conserved amongst all JSRV sequences. Furthermore, it displays a purportedly endogenous-specific *Hin*dIII site in *gag*, as well as the same endogenous locus 4-specific *Pst*I site which was found to be present in enJS5F16.

The only restriction marker that was consistently accurate amongst the sequences analysed here, was the differential *Sca*I site in *gag*, as published by Palmarini *et al.* (1996a). However, since the absence of this site was used as a criterion to select for the three endogenous loci, the results of the *Sca*I analysis are meaningless in this context. Furthermore, analysis of *gag* shows that the point mutation which results in the loss of the *Sca*I site in the endogenous sequences, is a silent mutation. Either sequence variation encodes glutamic acid at the altered codon, implying that the presence or absence of the *Sca*I site is of no functional significance.

### 2.3.3 Amplification of JSRV variable regions from goat DNA

To assess whether the three variable regions that had been identified for sheep betaretroviruses were also found in the endogenous betaretroviruses of goats, two sets of PCR primers were designed, based on consensus sequences of regions in the enJSRVs that were conserved upstream and downstream of the variable regions. VR1 and VR2 were amplified as one product using primers 533F and 1157R (numbering based on the sequence of $JSRV_{21}$). The forward primer 6748F anneals in the TM, upstream of VR3, and the reverse primer 35R anneals in part of the repeat region and U5 of the LTR, leading to amplification of part of TM (including VR3) and U3. Genomic DNA was isolated from cultured large T-immortalised goat embryo fibroblast (TIGEF) cells to provide template DNA for the PCR, and the resulting products were cloned using a TOPO TA Cloning Kit (Invitrogen). Two clones from each experiment were sequenced, and the sequences of the cloned inserts were incorporated into the alignments that had already been generated (Figures 2.14 and 2.15). They resemble the sheep endogenous loci in the three variable regions (Figures 2.9 and 2.12).

### 2.3.4 Phylogenetic analyses

All JSRV and JSRV-related nucleotide sequences available in Genbank by the time the three full-length endogenous sequences had been obtained, were assembled into a comprehensive alignment using the BioEdit programme in conjunction with ClustalW. Using selections from within this alignment, phylogenetic trees were generated for all genes, noncoding regions, and tandem genes / regions (*e.g.* U5 – 5' LTR – *gag*, *gag* – *pol*,

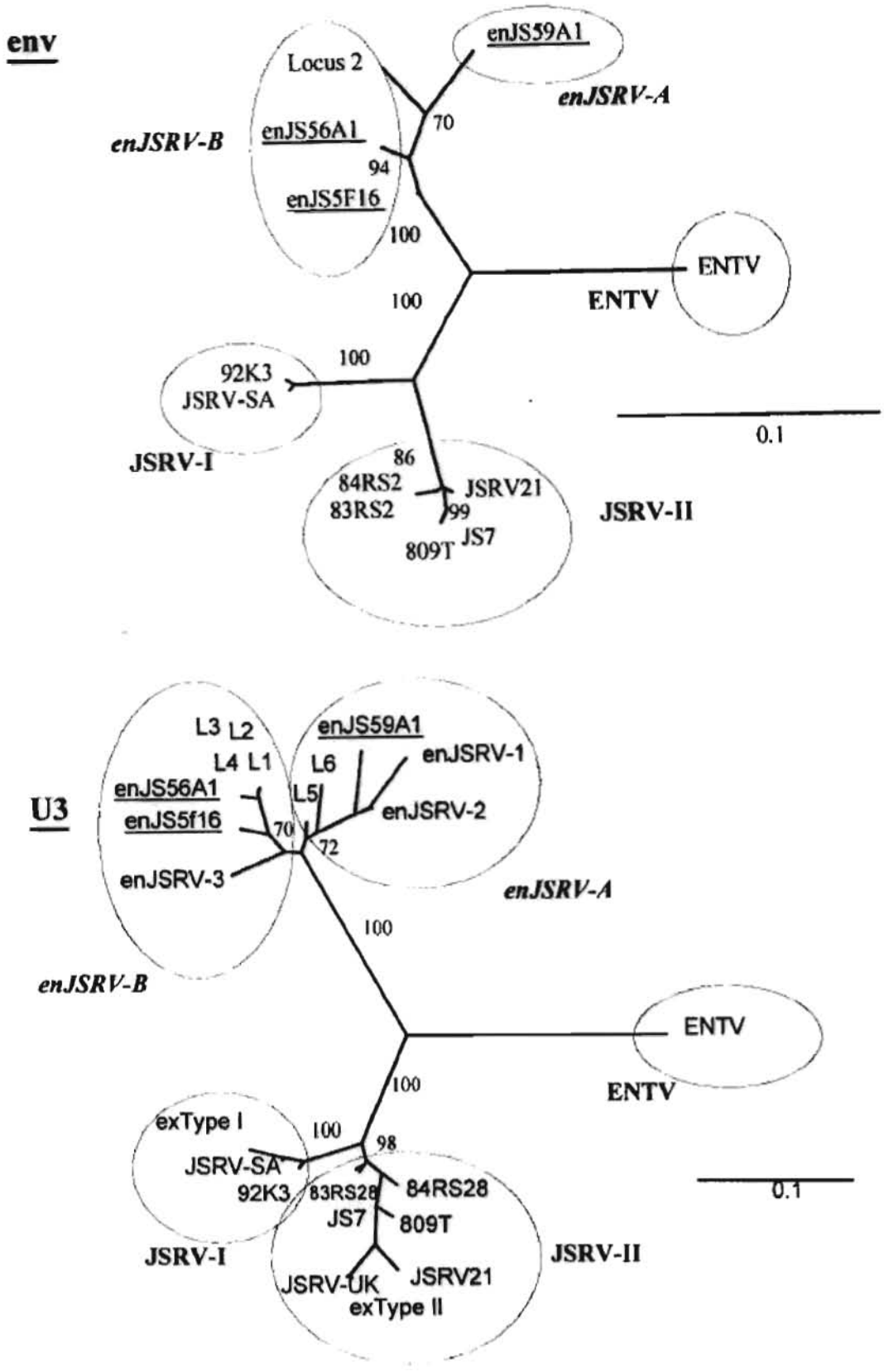



**Figure 2.13 Unrooted phylogenetic trees for *env* and U3 of ovine betaretroviruses, as derived by neighbour joining and 1,000 bootstrap sampling replications.** Endogenous loci 1-6 are abbreviated to L1 - L6. The branch lengths are proportional to an estimate of evolutionary change. Names of sequences generated in this study are underlined. Figures created by author for (Palmarini *et al.*, 2000b).

or *env* – U3), using programmes within the PHYLIP package, and TreeView. Three such trees were published (Palmarini *et al.*, 2000b), two of which are shown in Figure 2.13. In all the trees generated, three distinct branches could be discerned: one for endogenous loci, one for the exogenous ENTV sequence, and one for exogenous JSRV sequences. This trend was expected, and confirmed earlier findings based on limited *gag* sequences (Cousens *et al.*, 1996). The exogenous JSRVs could be seen to divide further into type I sequences of African origin and type II sequences of US and UK descent, as previously described (Bai *et al.*, 1996).

Another feature of all the trees, which had not previously been reported, was that enJS59A1 branched apart from the other two full-length endogenous loci cloned here. Other endogenous sequences previously isolated by PCR cloning consistently grouped with either enJS59A1 or with enJS5F16 and enJS56A1. This indicated at least two phylogenetic groups, which were termed enJSRV-A and enJSRV-B (Fig. 2.13). Additional confirmed enJSRV phylogenetic groups might arise if other complete proviral sequences become available.

The comprehensive alignment of all JSRV-related sequences was continually updated to include any new sequence data as they became available on Genbank. This alignment is available in electronic format (as a FASTA file) on request from the author. Once again drawing on this alignment and by incorporating all sequences that had become available since the first set of trees was published (Palmarini *et al.*, 2000b), phylogenetic trees were generated for the regions corresponding to the TM-U3 and *gag* goat DNA-derived PCR fragments (Section 2.3.3) (Figures 2.14 and 2.15). These later trees confirmed previous results pertaining to separate branches within exogenous JSRVs (JSRV-I and JSRV-II) and

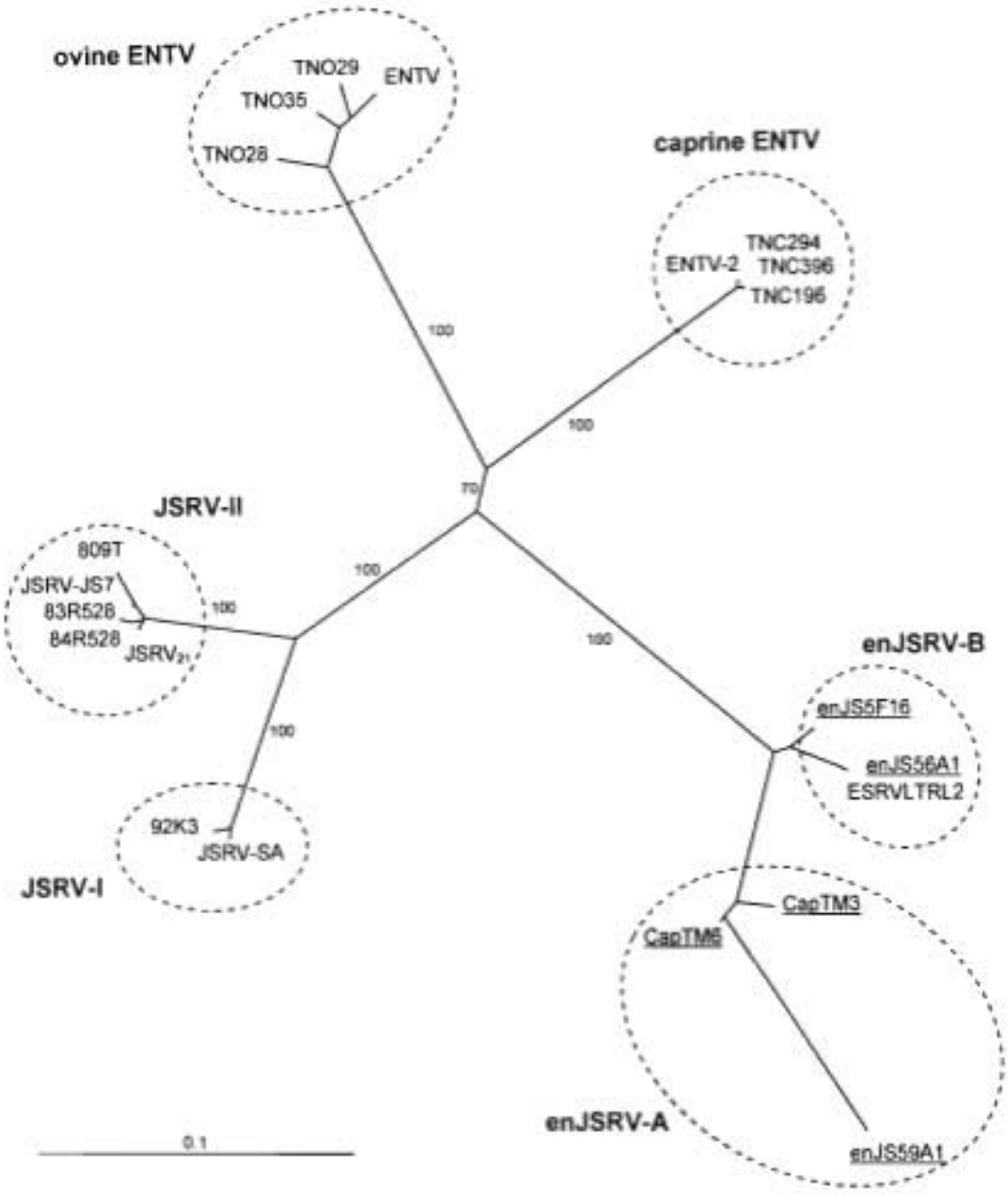



**Figure 2.14 Unrooted phylogenetic tree for TM-U3 of endogenous and exogenous ovine and caprine betaretroviruses, as derived by neighbour joining and 1,000 bootstrap sampling replications.** The branch lengths are proportional to an estimate of evolutionary change. Names of sequences generated in this study are underlined.

within endogenous JSRVs, and assigned the goat DNA-derived enJSRV fragments to the enJSRV-A group together with enJS59A1. Furthermore, they showed a clear phylogenetic grouping of ovine *vs.* caprine ENTV sequences, in keeping with descriptive comparisons of these sequences (Ortin *et al.*, 2003).

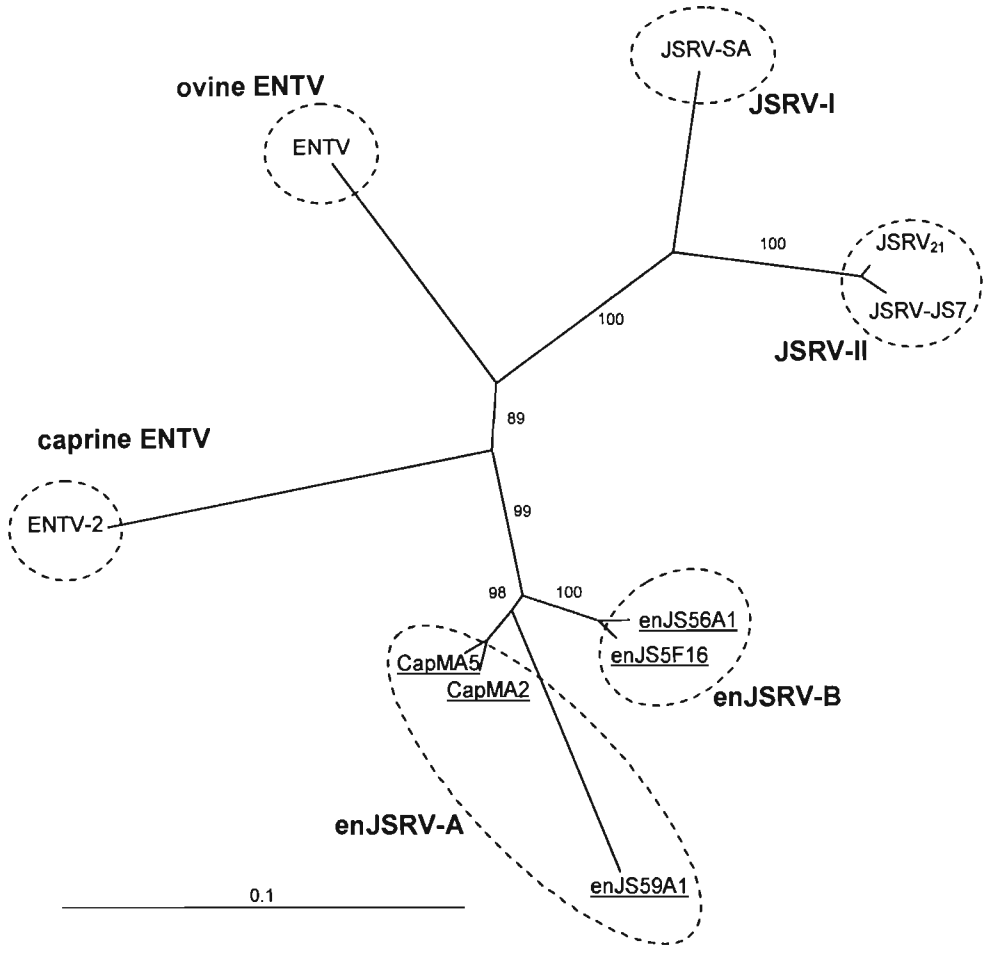



**Figure 2.15 Unrooted phylogenetic tree for part of *gag* of endogenous and exogenous ovine and caprine betaretroviruses, as derived by neighbour joining and 1,000 bootstrap sampling replications.** The branch lengths are proportional to an estimate of evolutionary change. Names of sequences generated in this study are underlined.

## 2.4 DISCUSSION

The overall aims of this part of the study were to fully sequence three full-length clones of endogenous, non-pathogenic JSRV, analyse and characterise the sequences obtained, and compare them to known exogenous, pathogenic JSRV sequences. Since no knowledge existed at the outset of this study that could relate any parts of the JSRV genome to its pathogenic potential, the sequence comparisons of the full-length enJSRVs to exogenous JSRV were to be carried out with a view towards identifying genes or genetic elements that consistently distinguish the non-pathogenic endogenous from the pathogenic exogenous isolates of JSRV. Such regions could then be implicated as potential factors involved in JSRV-mediated pathogenesis.

In short, the objectives of this study were met. For the first time, the complete nucleotide sequences of proviral endogenous JSRV loci were determined. Owing to the fact that the endogenous clones were obtained subsequent to endonuclease digestion of sheep genomic DNA, as opposed to being generated in fragments by PCR amplification, these sequences are a faithful representation of those found within the genetic material of the animals they were derived from. Analysis of the sequences revealed that they shared the same genomic organisation as exogenous JSRV. All three proviruses contained uninterrupted open reading frames for at least one structural gene. In particular, enJS56A1 was a virtually full-length provirus, with open reading frames for *gag*, *pro*, most of *pol*, and *env*. Sequence comparisons with exogenous JSRV showed that three short regions consistently distinguish enJSRVs from their exogenous counterparts. Two of these are in *gag* and one in the transmembrane region of *env*. These were called the three variable regions of

betaretroviruses of sheep and goats. Certain aspects of the study are discussed in detail in the following sections.

### 2.4.1 Sequencing of full-length endogenous JSRV loci

The elucidation of the three full-length enJSRV sequences presented a number of challenges, and took considerably more work and time to complete than an equivalent task would do, were it to be attempted now. One obvious reason for this is the progression of technology. That aside, the author had had no previous exposure to the field of bioinformatics, and relied for the entire duration of the sequencing part of this study on the software made available to him for the analysis and manipulation of the sequencing output data. This consisted of DNAsis for Windows, Version 2.1, and a DOS-based version of OLIGO for the design of primers. No software was used to visualise electronic versions of the electrofluorescent profiles, the use of which facilitates the viewing of sequence details. As described under Materials and Methods, the author was only provided with printouts of the electrofluorescent profiles, as well as the plain text files corresponding to the base calling of the software associated with the sequencer. All forms of sequence editing therefore had to be performed manually, which introduces a degree of subjectivity. This was compensated for by repeats of all sequences containing ambiguities, and sometimes by the design of new primers specifically based on the clones under study. Had software been available that allows one to view the raw data generated by the sequencer, the majority of the ambiguities could probably have been resolved by the identification of artefacts such as pull-up peaks. The author soon became very proficient at the visual editing of sequence

printouts, however. Whereas the first clone required nine months to sequence, the second sequence was completed in five months, and the third one in four.

Software limitations were not the only challenge posed to the sequencing aspects of the project. As described in section 4.1 under Results, the two sets of sequencing primers essentially constituted random primers, so no set strategy could be followed in their use. Having determined the positions where these primers anneal as part of sequencing the first clone, a more structured approach was applied to the sequencing of the other two clones. This, too, contributed to the shorter time frame in which their sequences were able to be determined.

The automated sequencer that the author had access to was committed to other applications two days per week, and when it was set up for sequencing, it was fitted with the shorter of the two available capillaries (length to detection window of 36 cm), thereby limiting the read length of the sequences. On those occasions when this sequencing facility returned several batches of unexpectedly failed results, the same samples were sent to another facility, which returned usable data. The alternative facility could not be accessed routinely owing to the substantially higher cost per reaction and its distance from our laboratory, but the usable read lengths per sequence were consistently 50 % to 65 % longer. This was attributable primarily to their use of the longer capillaries with a 50 cm length to the detection window. Availability of the long capillary throughout the sequencing aspect of the study would have reduced the total number of sequencing reactions required for each clone.

At the commencement of the study, the FS Terminator kit (PE Applied Biosystems), formally known as "ABI PRISM Dye Terminator Cycle Sequencing Core Kit With AmpliTaq DNA Polymerase, FS", was used. This was abandoned in favour of the Thermo Sequenase Dye Terminator Cycle Sequencing Premix Kit (Amersham) when the latter was introduced to the study. This was done because it yielded higher quality results, and the procedure required for cleaning up the sequencing reaction products prior to loading them on the automated sequencer was less time-consuming.

At a later point, PE Applied Biosystems introduced a product that was vastly improved with respect to the earlier FS Terminator kit, called the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit With AmpliTaq DNA Polymerase, FS. The BigDye kit compared favourably to the Thermo Sequenase kit in terms of sequence quality, and it was superior in terms of its ability to resolve G residues. It also yielded longer read lengths, and used less primer per reaction. Given that our primer stocks were limited to the aliquots donated to us by Dr. Palmarini, its use was considered a distinct advantage. Its drawbacks lay in the fact that the clean-up procedure was once again more time-consuming, and the cost per reaction was higher than for the Thermo Sequenase kit.

In an effort to lower the cost per reaction of the BigDye kit, half the BigDye Ready Reaction Mix was replaced with a buffer that was chemically equivalent to that of the Ready Reaction Mix, but lacking the polymerase enzyme, deoxyribonucleoside triphosphates and dye-labelled dideoxyribonucleoside triphosphate terminators. Comparative titration experiments showed that this dilution of active ingredients did not result in any marked deterioration of quality or read length relative to the use of undiluted

BigDye reactions. From this point, the use of Thermo Sequenase was abandoned entirely in favour of the BigDye kit.

### 2.4.2 Sequence analysis of full-length endogenous JSRV loci

The completion of the sequence of enJS5F16 constituted the first-ever sequence determination of a full-length endogenous JSRV. This was followed by the complete sequence determination of two further endogenous JSRV loci, which brought the number of completely sequenced endogenous betaretroviruses of sheep in line with the number of exogenous ones known at that point – JSRV-SA (York *et al.*, 1992), $JSRV_{21}$ (Palmarini *et al.*, 1999a) and ENTV (Cousens *et al.*, 1999). The approach that had been taken was superior to all previous attempts at elucidating the nucleotide sequences of enJSRVs, including the preliminary work carried out by the author for this study, wherein an entire enJSRV sequence had essentially been isolated by PCR cloning. However, since this approach necessitated the amplification of two fragments in order to assemble an almost full-length genome, one could not assume that these fragments were derived from the same locus, given that there are 15 to 20 enJSRV loci in the sheep genome.

The strengths of the approach taken in this study are, in essence, twofold. Firstly, the sequences are truly full-length and therefore represent entire genomic sequences. No PCR approach can lead to the amplification of proviral loci that would incorporate the entire LTRs on either end of the genome. Also, PCR is error-prone, especially when used to amplify long products such as full proviruses, even when using proof-reading *Taq* DNA polymerase enzymes (see Part II of this thesis). Such errors are of less significance when

the results are to be used for phylogenetic analyses, for instance, but could significantly confound any findings of functional studies.

The second strength of this study lay in the elucidation of three proviral endogenous genomic sequences, rather than just a single one. This permitted the meaningful comparison of the obtained sequences to define conserved characteristics and show which features are unique to individual loci, owing to mutations. It also allowed comprehensive comparisons with the exogenous JSRV and ENTV sequences to meet the most important objectives of this study – the identification of genetic elements that distinguish the non-pathogenic endogenous JSRVs from their pathogenic exogenous counterparts.

It is worth stating the obvious at this point, namely that the elucidation of full nucleotide sequences provides considerably more powerful data tools than relying on RE profiles to group and characterise sequences. This was aptly demonstrated by attempting to fit the three endogenous clones (as well as the exogenous $JSRV_{21}$) into an existing classification scheme for enJSRVs that had been developed based on RE profiling. $JSRV_{21}$ turned out to fit the profile of enJSRVs better than some of the endogenous clones did. This is no criticism of the previous study; the author appreciates the amount of work required to obtain sequence data. It merely reflects that the dividends of such work merit the expenditure, both in terms of time and funding.

#### 2.4.2.1 *orf-x*

The first noteworthy finding pertaining to the sequences of the endogenous loci was the fact that a large part of *pol* was deleted in enJS5F16. Such a deletion would likely render this locus entirely defective in terms of its ability to replicate, reverse transcribe or integrate independently. An endogenous retrovirus is derived at some point during the evolution of its host by integration of a functional retrovirus into the germ lines, causing fixation of the provirus. It was therefore not thought that the deletions in *pol* were likely to be characteristic of endogenous JSRVs in general. However, the contemporaneous 5' truncation of *orf-x* merited further investigation. Before completing the sequencing of enJS5F16, the part of *pol* that also contains *orf-x* was sequenced in enJS59A1 and found to be an uninterrupted open reading frame of the same length as in the exogenous JSRV sequences. *Orf-x* was consequently not considered a region that distinguishes endogenous from exogenous JSRVs.

#### 2.4.2.2 A full complement of ORFs in enJS56A1

When sequencing of the last clone, penJS56A1, was completed, it was noted that it encodes open reading frames for all structural JSRV genes. Its integrase domain at the 3' terminus of *pol* is truncated by 14 amino acids owing to a frameshift mutation, but this frameshift was not common to the other two loci. Therefore, enJSRVs were not found to be distinguished from JSRVs by consistently being defective in one or more genes. Such a conclusion would have been difficult to substantiate in any event, since the exogenous progenitors of enJSRVs must have been functional in their replicative and infectious

capacity, and unless they depended on helper viruses, would have had to encode a full complement of genes. The defects seen in the other two loci are, as with other endogenous retroviruses, most likely the effect of accumulated changes that occurred after their fixation in the sheep genome.

### 2.4.2.3 Evolutionary analysis of the enJSRV loci

Taking into account the four base pair differences between the 5' and 3' LTRs of enJS59A1, the two in enJS56A1, and the absolute sequence homology of the LTRs in enJS5F16, an estimate of the time of integration of these elements into the sheep germ line was made. At the time of integration, the LTRs of each provirus were presumably identical, since they would have arisen from sequence duplications at the 5' and 3' ends of the genome during reverse transcription. The rate of mutation of LTRs is presumed to equate to the general rate of mutation of noncoding sequences and pseudogenes. Thus, a molecular clock model was applied to the intragenomic variability of the LTRs to provide and estimate of the time elapsed since integration (Dangel *et al.*, 1995; Mager & Freeman, 1995; Medstrand & Mager, 1998). Calculating from an average value of $4.85 \times 10^{-9}$ substitutions per nucleotide site per year relative to pseudogenes (Li *et al.*, 1985), the integrations of enJS56A1 and enJS59A1 were estimated to have occurred approximately 0.9 and 1.8 million years ago, respectively, subject to a wide margin of error. Based on the sequence identity of the 5' and 3' LTRs in enJS5F16, this locus might have integrated less than 500,000 years ago.

These estimates concur with the conclusions of a previous study by Hecht *et al.* (1996b), wherein it was shown that the genomes of sheep (and wild members of the genus *Ovis*) and goats (and wild members of the genus *Capra*) each contain approximately 20 JSRV-related endogenous retroviruses. The RE profiles of these proviral elements differed between the two genera, but were similar among members of the same genus. This implies that at least the majority of enJSRVs were fixed in the germ line of sheep and goats after their speciation, which happened between four and ten million years ago (Irwin *et al.*, 1991). Thus, enJSRVs can be considered modern endogenous retroviruses, which is in keeping with their high degree of homology with the currently circulating exogenous retroviruses JSRV and ENTV (Boeke & Stoye, 1997).

This study also broadened the base of knowledge pertaining to the phylogenetic relationships amongst the currently known betaretroviruses of sheep and goats. Whereas some previous findings could be confirmed by the phylogenetic trees generated, the branching of ENTVs into those infecting goats and those infecting sheep, had not been graphically represented before, even though these groupings were described. An entirely novel finding in this study was the consistent branching of enJSRVs into two groups, akin to the division of exogenous JSRVs into type I and type II.

#### 2.4.2.4 The enJS56A1 genome does not support particle formation

As soon as the entire sequence of enJS56A1 was completed, the finding that it has open reading frames for all confirmed JSRV genes was communicated to our collaborators. Using *in situ* hybridization, they were in the process of showing that enJSRVs are most

highly expressed in the luminal epithelial (LE) and glandular epithelial (GE) cells of the ovine uterus (Palmarini *et al.*, 2000b). We had already raised the question of whether or not some enJSRVs might form particles *in vivo*, and they were well set up to test the ability

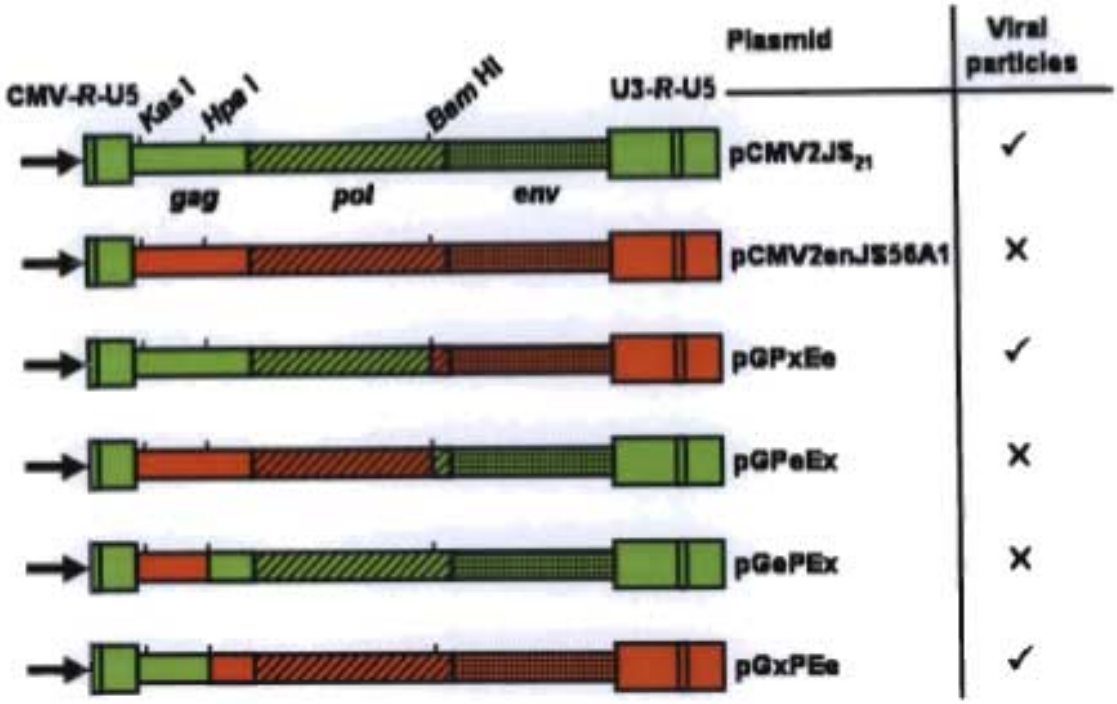



**Figure 2.16 Schematic of the CMV-driven chimeric constructs of $JSRV_{21}$ and enJS56A1 created by Palmarini *et al.* (2000b)(Palmarini *et al.*, 2000b) to narrow down the region responsible for the particle formation defect in enJS56A1.** The restriction endonuclease enzymes employed in the construction of the chimeras are indicated. The determinants of particle release were shown to reside in the amino-terminal two thirds of Gag.

of enJS56A1 to support particle formation *in vitro*, using the same system they had developed for $JSRV_{21}$ particle formation (Palmarini *et al.*, 1999a). The sequence of enJS56A1 was placed under the same transcriptional promoter system as the infectious molecular clone of $JSRV_{21}$ had been, giving rise to the expression construct Pcmv2enJS56A1. This was found not to support particle formation, as assayed by the release of mature capsid protein (a product of the *gag* gene) into the culture supernatant of transiently transfected 293T cells.

In order to localise the particle formation defect of pCMV2enJS56A1, a set of chimeric constructs was created (Palmarini *et al.*, 2000b) as outlined in Figure 2.16. These revealed that the only $JSRV_{21}$-derived region that is required for particle production is the amino-terminal two thirds of Gag (pGxPEe, where capital letters denote the genes *gag*, *pol* and *env*, and lower case letters denote either endogenous [e] or exogenous [x] origin). The remainder of the genome could be enJS56A1-derived without affecting particle production. The reciprocal chimera pGePEx was not able to support release of particles into the culture supernatant.

#### 2.4.2.5 Variable regions of ovine betaretroviruses in *gag*

The second principal goal of this aspect of the study was met when the variable regions in *gag* were discovered. These regions are highly conserved within the enJSRVs, including sequences derived from goat genomic DNAs. They are also conserved amongst the three known JSRV sequences JSRV-SA, $JSRV_{21}$ and JSRV-JS7. However, comparisons of VR1 and VR2 between enJSRVs, JSRVs and ENTV reveal a high degree of polymorphism. Of particular note is a stretch of seven consecutive proline residues found in VR1 of all JSRV sequences that is absent in enJSRVs.

The strict conservation of some sequence elements amongst both endogenous and exogenous JSRVs, such as the 35-bp stretch upstream of the *gag* start codon, imply that the function associated with these regions is common to all JSRVs. Such functions might relate to critical interactions with the host cell or to other invariable requirements of the viral life cycle. However, sequence elements that are conserved amongst only endogenous

or exogenous JSRVs, but differ between these two groups, serve to highlight regions that functionally distinguish exogenous from endogenous JSRVs, whether this relates to their potential role in pathogenesis, their host cell specificity, or other factors that discriminate between these two closely related groups of retroviruses. Since the role of the JSRV p15 Gag protein, which includes VR1 and VR2, is not known, it was not obvious which potential functions of these regions could be investigated. Although the significance of the protein(s) between MA and CA was generally poorly understood, these regions had been demonstrated to play roles in particle assembly and budding in some retroviruses (Vogt, 1997b).

It was therefore of particular interest that the region identified by the construction of the $JSRV_{21}$ / enJS56A1 chimeric clones as harbouring the endogenous particle release defect encompassed both VR1 and VR2. The likelihood that either of the variable regions, or their combined presence, is responsible for the observed defect seemed very high, in view of the high degree of homology between endogenous and exogenous JSRVs in *gag* outside of the variable regions. However, allowance was made for the possibility that some single nucleotide difference(s) in the coding region of *gag*, or in the untranslated leader region of *gag*, determines the particle formation defect.

#### 2.4.2.6 The variable region of ovine betaretroviruses in *env*

Although a high degree of polymorphism observed amongst JSRV-related sequences in the carboxy-terminal end of the transmembrane portion of *env* had been reported previously (Bai *et al.*, 1999; Cousens *et al.*, 1999), it had not been formally established as a feature

that distinguishes pathogenic exogenous from non-pathogenic endogenous sequences. It was named the variable region 3 (VR3) of ovine betaretroviruses as a result of this study.

The author considered it more likely that VR3 would have a direct involvement in JSRV pathogenesis than either VR1 or VR2, since the potential opportunities of envelope protein components interacting with cellular signalling pathways are more obvious than those of Gag components. Also, a report had just become available that linked a retroviral envelope gene to cell proliferation and anchorage-independent growth of some epithelial and fibroblast cell lines *in vitro* (Alian *et al.*, 2000). Since our collaborators at the CRI/UCI had the relevant facilities and expertise for the investigation of the possible involvement of JSRV Env in transformation, it was decided that they focus on that aspect, while the author developed the relevant molecular tools for the functional characterisation of VR1 and VR2.

### 2.4.3 Applications of the sequence analyses

The comprehensive sequence alignment maintained by the author can potentially be put to good use in the design of highly specific PCR primers. The author designed a VR1-based primer specific for exogenous JSRV sequences that, in combination with a primer that would anneal to all known JSRV-related sequences, allows more specific detection of exogenous JSRV against a high background of enJSRVs than other methods that rely on a single PCR (as opposed to nested or hemi-nested PCR designs) (Padayachi, 2005).

Another application was in the design of LTR-based enJSRV-specific primers by the author. This was done with a view towards conducting a broad and comprehensive phylogenetic study of endogenous JSRV-related loci in wild ungulates (hoofed animals). Regrettably, the group that we established a collaboration with to obtain all the samples required for the study never provided a single sample. However, the amplification of near full-length enJSRVs first from plasmid clones, and then from sheep DNA was successfully optimised, despite the high background of shorter and presumably highly defective enJSRVs.

# CHAPTER 3

# FUNCTIONAL ANALYSIS OF JSRV VARIABLE REGIONS

## 3.1 INTRODUCTION

In addition to providing relevant insights into the evolutionary biology of enJSRVs and the degree and nature of their relationship to exogenous JSRV and ENTV isolates, the first phase of the study described in this thesis identified three short variable regions in the known betaretroviruses of sheep and goats. This represented a milestone in the field, as these variable regions were seen to be the defining genetic elements that distinguish pathogenic exogenous from non-pathogenic endogenous JSRV sequences. Upon publication of these findings (Palmarini *et al.*, 2000b), the task of characterising the three variable regions was divided between our laboratory and that of our collaborators at the CRI/UCI in accordance with the facilities and expertise available at each institution. We did not have the requisite facilities to work on *env* and VR3, whereas the CRI/UCI did. Furthermore, the author was considered by his colleagues to have the skill and patience required to design and develop the relevant molecular tools that would enable the comparative study of endogenous *vs.* exogenous VR1 and VR2, partly in an attempt to follow up on the particle release defect of enJS56A1 (Palmarini *et al.*, 2000b). The *in vitro* study of JSRV *env* and VR3 required the direct input of a far greater number of researchers than we would have been able to dedicate to the project, even if we had managed to put all the relevant facilities in place.

The study of JSRV *env* commenced at the same time as the author's study of VR1 and VR2, and there was little *a priori* indication to suggest that either avenue would provide more pertinent insights into JSRV pathogenesis than the other. Since the author's study formed part of a broad, international research endeavour to study the pathogenesis of JSRV and to define the applicability of the OPA model for human carcinomas, he was required, as part of his work, to critically assess all the developments pertaining to JSRV *env* and VR3. The objectives of this aspect of the study were thus two-tiered. The first was to develop molecular tools that could be used, in future, to discern the biological significance of VR1 and VR2 *in vivo*. These tools were required to be designed, constructed, verified, and finally assessed *in vitro* for their applicability to *in vivo* studies. The second tier of this part of the study was to maintain a thorough perspective on the developments in JSRV *env* and VR3 progress in order to put both parts into the context of the overall aims of JSRV pathogenesis research.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Techniques employed

Techniques used for this aspect of the study, which were not already described in Chapter 2, are outlined below.

#### 3.2.1.1 Restriction endonuclease digestions

Restriction endonuclease (RE) digestions were performed either at 37°C for two hours, or at 16°C overnight in 50 µl total reaction volumes, using 4 units of each enzyme. Reactions were supplemented with a final concentration of 100 µg/ml Bovine Serum Albumin. DNA substrate was added at ~100 ng per reaction, of either plasmid DNA or PCR product. The following REs were used:

*Hpa*I (3 - 10 U/µl; Roche)

*Pac*I (10 U/µl; New England Biolabs [NEB])

*Sex*AI (4 U/µl; NEB)

*Sma*I (10 U/µl; Roche)

*Xba*I (10 U/µl; Roche)

After optimisation all digests, including double-digests using two NEB-sourced enzymes, were carried out in 1× SuRE/Cut Buffer A (Roche). This was due to the fact that NEBuffer 1 supplied with *Pac*I is not compatible with *Sex*AI activity, and NEBuffer 3 supplied with *Sex*AI is incompatible with *Pac*I. All digests worked optimally in SuRE/Cut Buffer A, however.

### 3.2.1.2 Purification of PCR products and agarose gel excised DNA fragments

The High Pure PCR Product Purification Kit (Roche) was used to remove contaminating substances such as unincorporated dNTPs, primers and salts from the PCR products. The technique involved addition of the chaotropic salt guanidine thiocyanate, present within the binding buffer, to PCR amplified DNA. The DNA binds selectively to special glass fibres filled within the High Pure filter tube.

Procedure:

- The desired band was excised from an agarose gel using a scalpel blade, under UV transillumination. The appropriate eye protection and gloves were used.
- The piece of agarose containing the DNA band of interest was placed into a 1.5 ml microcentrifuge tube.
- The gel mass was determined by weighing the microcentrifuge tube prior to addition of the gel segment and after the gel piece was added to the tube using a coarse balance.
- A volume of 300 µl binding buffer was added to a microcentrifuge tube for every 100 mg of gel segment.
- The microcentrifuge tube containing the agarose piece was vortexed for 30 seconds to resuspend the gel segment in the binding buffer.
- The gel mixture was heated at 56°C for 10 minutes to liberate the DNA by dissolving the agarose. The suspension was vortexed using short bursts every 2-3 minutes during the incubation period.
- After incubation, the DNA was precipitated by addition of 150 µl isopropanol per 100 mg gel segment, into the microcentrifuge tube.

- The tube was thoroughly vortexed for 3-5 seconds.
- A High Pure filter tube was inserted into a collection tube.
- The mixture was carefully transferred to the upper reservoir of the filter tube.
- A maximum volume of 700 µl was added at each time. (If there were more than 700 µl, the procedure was completed to the end of the elution step. Thereafter, the filter was re-used to bind and elute the second 700 µl volume to ensure that the binding capacity of the filter was not compromised by the addition of excess DNA).
- Centrifuged for 60 seconds at maximum speed in a table top microcentrifuge at room temperature.
- The filter tube was disconnected and the flow-through was discarded.
- Re-connected the filter tube to the collection tube.
- Added 500 µl wash buffer to the upper reservoir.
- Centrifuged for 60 seconds at maximum speed.
- Discarded the flow-through solution and collection tube.
- Re-connected the filter tube with the same collection tube.
- Added 200 µl wash buffer.
- Centrifuged for one minute at maximum speed.
- Discarded the flow-through solution and collection tube
- Centrifuged for 30 seconds at maximum speed to remove residual wash buffer.
- Discarded the flow-through and collection tube.
- Re-connected the filter tube to a clean 1.5 ml microcentrifuge tube.
- Added 50-100 µl $dd_iH_2O$ to the upper reservoir of the filter tube depending on the expected DNA yield.
- Centrifuged for one minute at maximum speed to collect eluate.

The same kit was used to purify PCR products prior to RE digestion. The following procedure was followed:

- To a 1.5 ml microcentrifuge tube containing 100 of DNA, 500µl of binding was added (binding buffer was adjusted according to the amount of DNA that was present).
- The mixture was thoroughly vortexed for three seconds.
- A High Pure filter tube was inserted into a collection tube.
- The mixture was carefully transferred to the upper reservoir of the filter tube.
- Centrifuged for 60 seconds at maximum speed in a table top microcentrifuge.
- The filter tube was disconnected and the flow-through was discarded.
- Re-connected the filter tube to the collection tube.
- Added 500 µl wash buffer to the upper reservoir.
- Centrifuged for 60 seconds at maximum speed.
- Discarded the flow-through solution and collection tube.
- Re-connected the filter tube with the same collection tube.
- Added 200 µl wash buffer.
- Centrifuged for one minute at maximum speed.
- Discarded the flow-through solution and collection tube
- Centrifuged for 30 seconds at maximum speed to remove residual wash buffer.
- Discarded the flow-through and collection tube.
- Re-connected the filter tube to a clean 1.5 ml microcentrifuge tube.
- Added 50-100 µl $dd_iH_2O$ to the upper reservoir of the filter tube depending on the DNA that was initially added.
- Centrifuged for one minute at maximum speed to collect eluate.

### 3.2.1.3 Sodium acetate / ethanol precipitation of DNA

RE-digested DNA was precipitated prior to ligation, using the following procedure: To a 1.5 ml microcentrifuge tube containing both double-digested plasmid and double-digested PCR fragment DNA in solution, 0.1 volumes of sodium acetate (3 M; pH 5.2) and 2.5 volumes absolute ethanol were added. The contents were mixed by vortexing and kept at -20°C for one hour. DNA was precipitated by centrifuging in a microcentrifuge in the cold room for ten minutes at top speed (16,000 × *g*). The supernatant was aspirated and the DNA pellet was washed by adding three volumes (relative to the original volume of DNA) of cold 70% ethanol, vortexing, and centrifuging for five minutes, as above. Air-dried DNA pellets were used in the subsequent ligation steps.

### 3.2.1.4 Ligation of restriction endonuclease digested DNA

Ligation reactions were set up by adding the following to the pellets comprised of double-digested DNA:

| | |
|---|---|
| $dd_iH_2O$ | 16 µl |
| T4 DNA ligase (1 U/µl; Roche) | 2 µl |
| 10× ligase buffer containing ATP | 2 µl |

The contents were mixed by vortexing to reconstitute the DNA in the ligation mix. This was left to incubate overnight at 4°C.

### 3.2.1.5 Boiling method for direct colony PCR

The following procedure was employed to obtain small amounts of plasmid DNA from transformed *E. coli* colonies, to test if they contained desired inserts:

Using a 200 µl pipette tip, individual transformed colonies were picked from transformation plates. The pipette tips containing the bacterial cells were touched to labelled positions on reference agar plates, and the remaining cells were resuspended in 1.5 ml microcentrifuge tubes containing 50 µl $dd_iH_2O$. The tubes were vortexed, closed and placed in a heating block at 100°C for five minutes. They were subsequently placed on ice for two minutes to precipitate moisture on the insides of the tubes. Cell debris was precipitated by centrifugation in a microcentrifuge at maximum speed for five minutes. Of the supernatant, five microlitres were used as template in PCRs.

### 3.2.1.6 Mammalian cell tissue culture

All cell culture experiments were carried out using aseptic techniques in a Class IIB biohazard laminar flow cabinet installed with HEPA filters. Human 293T immortalised fibroblasts and sheep choroid plexus (CP) cells were incubated under standard cell culture conditions comprising 5% $CO_2$ in a 100% humidified, water-jacketed incubator adjusted to 37°C. Cells were grown in 10 cm tissue culture dishes using Dulbecco's modification of Eagle's medium (DMEM; Cellgro) containing 45 g/l glucose and L-glutamine. DMEM was supplemented with 10% foetal bovine serum (FBS; Gibco BRL). Bacterial contamination was prevented by the addition of 1,000 iU/ml penicillin to a final

concentration of 100 iU/ml and 1,000 μg/ml streptomycin to a final concentration of 100 μg/ml. Cell growth was observed using an inverted light microscope under phase contrast. 293T cells were split 1:6 when they reached 80% confluence, using 0.25% trypsin to enzymatically facilitate detachment of the cells from the culture dish. CP cells were also split at 80% confluence, but only 1:4.

### 3.2.1.7 Transfection of human 293T cells

293T cells were trypsin neutralised in regular growth media and seeded into either 10 cm or 15 cm culture dishes depending on the transfections being performed. The cells were transfected at 50% confluence with either 21 μg or 28 μg plasmid DNA in 10 cm dishes or with 58 μg DNA in 15-cm dishes using the CalPhos Mammalian Transfection kit (Clontech), following the protocol outlined in the manufacturer's handbook. The growth medium for each culture dish was replaced 12-16 hours post-transfection with 5 ml fresh medium for 10 cm dishes and with 12.5 ml for 15 cm dishes. The medium was then harvested and replaced at 24, 48 and 72 hours after the initial medium change. The 24 and 48 hour culture supernatant fractions were stored at -70°C until the 72 hour fraction was collected.

### 3.2.1.8 Concentration of viral particles

Post-transfection culture supernatants from 24, 48 and 72 hours after the initial change of medium were pooled and centrifuged using a benchtop centrifuge at 3,000 × *g* for 10-15

minutes at 4°C to remove residual cell debris. The pooled media were filtered using 0.45 μm pore size, sterile filters. Viral particles were pelleted by ultracentrifugation in a Beckman SW28 rotor over a double glycerol cushion as follows:

Clear-walled ultracentrifuge tubes received 3 ml each of 50% glycerol, made up in TNE buffer [100 mM NaCl, 10 mM Tris, 1 mM EDTA; pH 7.4]. A further 3 ml of 25% glycerol (made up in TNE) was carefully added drop-wise to complete the double glycerol cushion. Clarified medium was gently added to each tube, held at an angle of ~30° so as not to disrupt the glycerol cushion. Ultracentrifugation was carried out at 100,000 × *g* for one hour at 4°C. The liquid was decanted from the tubes and the viral pellets were resuspended in TNE buffer at 300 or 400-fold higher concentration relative to the initial supernatant and stored at –70°C.

### 3.2.1.9 Western blotting

Reagents used:

- Laemmli electrode buffer (5 litre - a 10× stock of 500 ml was made)

  0.25 M Tris

  1.92 M glycine

  1% (w/v) SDS

  It was a requirement that the pH not be adjusted.

- Transfer buffer (2 litres – a 10× stock of 500 ml was made)

  0.02 M Tris, pH 8.3

  0.15 M Glycine

  20% Methanol

- Blocking buffer (45 ml)

| | |
|---|---|
| 5% (w/v) non-fat dry milk | 2.25 g |
| TBS + 0.1% Tween-20 | 45 ml |

  10 ml blocking buffer was added per blot.

- 10× Tris-buffered saline (TBS, 1 litre)

| | |
|---|---|
| 0.2 M Tris | 24.2 g |
| 50 mM NaCl | 80.0 g |
| Adjusted pH to 7.6 | |

- Wash buffer (1 litre)

  1× TBS

  0.05% Tween-20

Gels made:

- 10% resolving gel

| | |
|---|---|
| Sucrose | 1 g |
| 40% acrylamide | 5 ml |
| 4× resolving buffer | 5 ml |
| $dd_iH_2O$ | 9 ml |
| TEMED | 20 µl |
| 10% (w/v) APS | 100 µl |

The TEMED was added last to the mixture as it serves as the polymerising agent.

The APS could be stored for a maximum of two weeks at 4°C.

- 3% Stacking gel

| | |
|---|---|
| 40% acrylamide | 750 µl |
| 2× stacking gel buffer | 5 ml |
| $dd_iH_2O$ | 4 ml |
| TEMED | 10 µl |
| 10% APS | 50 µl |

The gels were poured and left to set for at least one hour before the samples were loaded.

Buffers used:

- 4× resolving buffer

| | |
|---|---|
| 1.5 M Tris, pH 8.8 | 36.34 g |
| 0.4% SDS | 8 ml (10% SDS stock) |

- 2× stacking buffer

| | |
|---|---|
| 0.25 M Tris, pH 6.8 | 3.30 g |
| 0.2% SDS | 2 ml (10% SDS stock) |

The polyacrylamide gel electrophoresis (PAGE) apparatus was assembled as follows:
Two glass plates were chosen, with the one being smaller than the other. The glass plates were cleaned with detergent and subsequently rinsed with 100% ethanol.
Two spacers (0.75 mm) and clamps were used to hold up the gels.
A 0.75 mm eight-well comb was used.

After the apparatus was assembled, the gels were prepared as follows:

- The resolving gel was mixed. Upon addition of the TEMED, the gel was loaded using a needle and syringe to ±1 cm below the lower edge of where the comb would reach.
- The stacking gel was loaded above the resolving gel and the comb inserted between the plates.
- The gel was left to polymerise for one hour.

- The gel was transferred to the running tank. The tank was filled with Laemmli electrode buffer. The wells of the gel were washed with Laemmli buffer using a syringe.
- A 1× solution of loading dye was made with Laemmli buffer. A volume of 1-2 µl was added to each well to make them visible for sample loading.
- The retroviral particles (in TNE buffer) were mixed with loading dye and subsequently boiled at 95°C for five minutes. Samples were placed on ice for two minutes and briefly centrifuged to collect the contents at the bottom of the tube.
- A 6 µl volume of the broad range protein marker (New England Biolabs) was loaded into the first well.
- A total volume of 20 µl (including the loading dye) was loaded into each of the other wells.
- The gel was electrophoresed at 70 V for 30 minutes to allow the sample contents to collect at the bottom of the stacking gel.
- The potential difference was then increased to 100 V and the gel was electrophoresed until the tracking dye ran off the gel.
- After electrophoresis, the gel was transferred to a Hybond nitrocellulose membrane using the sandwich cassette system from Amersham Biosciences.
- The transfer tank was kept cold by inserting the tank into an ice bucket containing crushed ice.
- The Tris-glycine containing transfer buffer was added to the buffer tank and the transfer was performed at 100 V for one hour.
- The sandwich cassette was then dismantled and the nitrocellulose membrane was placed in TBS overnight at 4°C. Care was taken not to touch the membrane, therefore forceps were used at all times.

- Subsequently, the nitrocellulose membrane was blocked for one hour in blocking buffer.
- The membrane was probed using an anti-JSRV *gag* polyclonal antibody for one hour at room temperature. A 1:500 dilution of the antibody was used in a volume of 8 ml wash buffer.
- Following primary antibody incubation, the membrane was washed three times for 10 minutes each.
- The membrane was then placed into a 1:1000 dilution of goat-anti-rabbit IgG (ImmunoPure) secondary antibody labelled with horseradish peroxidase. The secondary antibody was diluted in wash buffer.
- The incubation period of the membrane was one hour at room temperature.

Prior to detection, the membrane was washed in wash buffer three times for 10 minutes each. Detection was performed using the Super Signal West Pico Chemiluminescent kit (Pierce) by adding 3 ml Super Signal chemiluminescent substrate stable peroxide solution to 3 ml Super Signal chemiluminescent substrate luminal enhancer. The membrane was incubated in the solution for 2-5 minutes in the dark. The membrane was transferred to a plastic surface and used to expose an X-ray film for a period of 2-4 minutes and development was carried out manually by immersion in developing, fixing and washing solutions.

### 3.2.1.10 RNA extraction from concentrated retroviral particles

Particles were resuspended in TNE buffer, so the standard reaction conditions of downstream applications had to be adjusted to suit DNase I digestion. A 1× solution of phosphate-buffered saline (PBS) containing 24 mM $MgCl_2$ was used in the procedure to compensate for the ETDA in TNE buffer. This served as a 2× diluent for a final $MgCl_2$ concentration of 12 mM. A 500 µl volume of PBS + 24 mM $MgCl_2$ was made. A 0.5 µl volume (5 U) of RNase-free DNase I (Roche) was used to make the DNase mix. Throughout the procedure, 2 ml screw cap tubes were used.

- For each sample, 200 µl of resuspended viral pellet was added to a 200 µl volume of DNase mix.
- The suspension was incubated at 37°C for 30 minutes.
- To the suspension, three volumes (1.2 ml) TRIzol LS were added. TRIzol LS is a noxious chemical therefore all steps involving its use were carried out in a fume hood.
- The tube contents were mixed by vortexing.
- The homogenised samples were incubated for five minutes at room temperature.
- Chloroform (without isoamyl alcohol) was added to the tube contents at 0.8 volumes of the original sample (320 µl).
- The tube was shaken vigorously by hand for 15 seconds and subsequently incubated for 10 minutes at room temperature.
- The tube was centrifuged at ≤ 12,000 × *g* for 15 minutes at 4°C. Samples separated into a lower red organic phase, an interphase and an upper colourless aqueous phase. The upper aqueous phase constituted 70% of the volume of TRIzol added (840 µl).

- The aqueous phase was transferred to a new 2.0 ml tube.
- A 0.5 µl volume of 20 mg/ml glycogen and two volumes of isopropanol relative to the original sample of 800 µl were added to the tube.
- The mixture was vortexed and left to incubate at room temperature for 10 minutes.
- After incubation, it was centrifuged at ≤ 12,000 × *g* for 10 minutes at 4°C.
- The supernatant was removed and the pellet was washed with four volumes (1.6 ml) of 75% ethanol, which was made up with DEPC-treated $dd_iH_2O$.
- The sample was mixed by vortexing, and centrifuged at ≤ 7,500 × *g* for five minutes at 4°C.
- The pellet was air dried for 5-10 minutes.
- The pellet was resuspended in a 60 µl volume of DNase reaction mix comprising 1 × DNase I buffer (Invitrogen) made up in DEPC-treated $dd_iH_2O$ and 0.2 U RNase-free DNase I (Roche).
- The mixture was incubated at room temperature for 15 minutes.
- DNase I was inactivated by the addition of 6 µl 25 mM EDTA and subsequent heating at 65°C for 10 minutes.
- The RNA was stored at -70°C.

#### 3.2.1.11 *In vitro* infection of ovine choroid plexus cells

Infectivity assays of the three chimeric clones were carried out according to a modification of the protocol developed for the *in vitro* infection of ovine cell lines by the CRI/UCI (Palmarini *et al.*, 1999b). Four infections were carried out, with concentrated viral particles derived from $pCMV2JS_{21}$ (as a positive control) and from the three chimeric clones. Each

infection required two 5 cm tissue culture dishes with CP cells at 50% confluence. The viral pellets (~450 µl) were made up to two millilitres with DMEM, supplemented as described earlier for tissue culture, as well as with 8 µg of Polybrene per ml. [Polybrene is a small cationic polymer that binds to cell surfaces and neutralizes surface charge, thereby allowing the viral glycoproteins to bind more efficiently to their receptors, because it reduces the repulsion between sialic acid-containing molecules.] The particle suspensions were passed through 0.2 µm filters and then divided in half. One half was incubated at 65°C for 15 minutes to inactivate enveloped retroviral particles. Culture media were aspirated from the CP cells and replaced with particle suspensions (at this point, 1 ml each). The cells were left to incubate for two hours at 37°C, where after 2 ml medium containing 8 µg/ml of Polybrene were added to each plate. After 16 hours, the particle-containing media were aspirated and replaced with 5 ml medium per plate, containing Polybrene at a concentration of 2 µg/ml. This concentration of Polybrene was subsequently maintained as the cells were passaged.

### 3.2.1.12 DNA extraction from cultured mammalian cells

DNA was isolated from sheep choroid plexus cells using the DNeasy Tissue kit (Qiagen). The principle of this kit is based on selective binding of DNA to a silica-gel-membrane. This technologically improved method produces purified DNA in the absence of noxious organic chemicals and ethanol precipitation.

Before the procedure was undertaken, the following instructions as outlined in the manufacturer's handbook were adhered to:

- Buffer AL was inspected carefully for the presence of a precipitate. If present, the precipitate was re-dissolved by incubation at 55°C.
- Buffers AW1 and AW2 were diluted in the appropriate amounts of 100% ethanol as indicated on the bottles, as they were supplied in a concentrated form.
- A water bath or heating block was set to 70°C before the procedure was carried out.
- Centrifugation steps were performed at room temperature using a speed of ≥6,000 × *g*.
- The pulse vortexing method comprising 5-10 seconds was used throughout the procedure.
- For RNA-free DNA, RNase A was added.
- Sterile, autoclaved PBS was prepared for use in the procedure.

Procedure:

- Choroid plexus cells were removed from the 37°C cell culture incubator and washed with PBS to remove any residual debris and dead cells. The PBS was aspirated. A volume of 500 µl PBS was added to the cell culture dish. A cell scraper was used to scrape cells off the surface of the culture plate. The cells, together with the PBS were placed into a 1.5 ml microcentrifuge tube. The cells were centrifuged for five minutes at 300 × *g*. The resulting pellet was reconstituted in 200 µl PBS.
- A 4 µl volume of RNase A at a concentration of 100 mg/ml was added to the resuspended DNA to remove any contaminating RNA from the suspension. The cells were incubated in RNase A for two minutes at room temperature.
- Contaminating protein complexes were degraded by the addition of 20 µl proteinase K. Thereafter, Buffer AL was added to the cells at a volume of 200 µl. The

suspension was mixed thoroughly by pulse vortexing to create a homogeneous solution. The cells were then incubated at 70°C for 10 minutes.

- Following incubation, 200 µl of 100% ethanol was added to the cell suspension. The sample was mixed thoroughly by vortexing. (The addition of ethanol could yield a white precipitate, which was added to the spin column as it does not interfere with the procedure).
- The entire cell suspension contents within the microcentrifuge tube were transferred to the upper part of the DNeasy spin column, which was housed within a 2 ml collection tube. The sample was centrifuged at ≥ 6,000 × *g* (8,000 rpm) in a bench top microcentrifuge for one minute. The collection tube containing contaminating cell debris was discarded.
- A new collection tube was used to house the spin column containing the bound DNA. The attached DNA was washed by the addition of 500 µl Buffer AW1 to the centre of the membrane. This was followed by centrifugation at ≥ 6,000 × *g* (8,000 rpm) for one minute. The collection tube containing the flow-through was discarded to prevent any carry-over contamination.
- The spin column containing the attached DNA was placed into a new collection tube. A 500 µl volume of Buffer AW2 was added to the DNA bound to the membrane, followed by centrifugation for three minutes at maximum speed. This wash buffer contains ethanol, therefore the membrane was required to be dried (hence the need for the three minute centrifugation at maximum speed). The presence of ethanol would have inhibited downstream reactions such as PCR procedures, thus all precautions were taken to prevent carry over of ethanol into the subsequent elution step. Following centrifugation, the flow though was discarded and the collection tube was re-inserted into the spin column.

- As an amendment to the procedure in the manufacturer's handbook, a further one minute centrifugation step at maximum speed was included in the absence of liquid in the collection tube. The handbook suggested this additional centrifugation only if the spin column made contact with the flow-through. However, on account of the author's previous experience with spin column procedures, this step was incorporated into the procedure. The author has noted from other such spin column procedures that discarding the flow-through from the initial centrifugation step facilitated the removal of additional ethanol and in so doing pure, ethanol free DNA was obtained.
- A sterile 1.5 ml microcentrifuge tube was used to house the spin column. A 200 µl volume of Buffer AE was carefully added to the centre of the DNeasy membrane. Care was taken to ensure that droplets of elution buffer did not touch the sides of the tube as this would prevent optimal elution of the bound DNA. The spin column was incubated at room temperature for one minute and subsequently centrifuged for one minute at ≥ 6,000 × *g* (8,000 rpm) to effect elution.
- The elution step was repeated by placing the spin column into a new, sterile microcentrifuge tube. A further 200 µl elution Buffer AE was placed into the centre of the membrane. The DNA containing spin column was incubated for one minute at room temperature as above. After incubation, the DNA was eluted by a one minute centrifugation at ≥ 6,000 × *g* (8,000 rpm). The 200 µl elution volume was optimal for the procedure since an increase in the volume would have resulted in the spin column making contact with the eluate owing to the funnel-shaped nature of the microcentrifuge tube.

### 3.2.2 Substitution of VR1 and VR2

The creation of chimeric constructs based on pCMV2JS$_{21}$ containing the endogenous (penJS56A1-derived) forms of VR1 and VR2 in place of their exogenous counterparts, either individually or in combination, was a multi-step process based on the principle of overlap extension PCR (Higuchi *et al.*, 1988; Ho *et al.*, 1989). The basic experimental steps involved were as follows:

- Creation of PCR products that, when combined as templates in further PCRs, would yield pCMV2JS$_{21}$-based amplicons with penJS56A1-derived VR1 or VR2.
- Restriction of the PCR amplicons and the target pCMV2JS$_{21}$ backbone with REs on either side of the substituted regions.
- Ligation of the fragments containing the penJS56A1-derived variable regions into the pCMV2JS$_{21}$ backbone.
- Transformation of competent *E. coli* with the resulting chimeric plasmid.
- PCR-screening of the transformed bacterial colonies and selection of those containing correctly sized inserts.
- Culture amplification of the relevant clones and confirmation by DNA sequencing.

#### 3.2.2.1 Building the method

The author began by developing a conceptual framework of how the penJS56A1-derived VR1 could be precisely substituted into the pCMV2JS$_{21}$ backbone. A model of how a PCR amplicon based on pCMV2JS$_{21}$, but containing the VR1 of penJS56A1, could theoretically be created, was developed and is graphically represented in Figure 3.1. This model draws

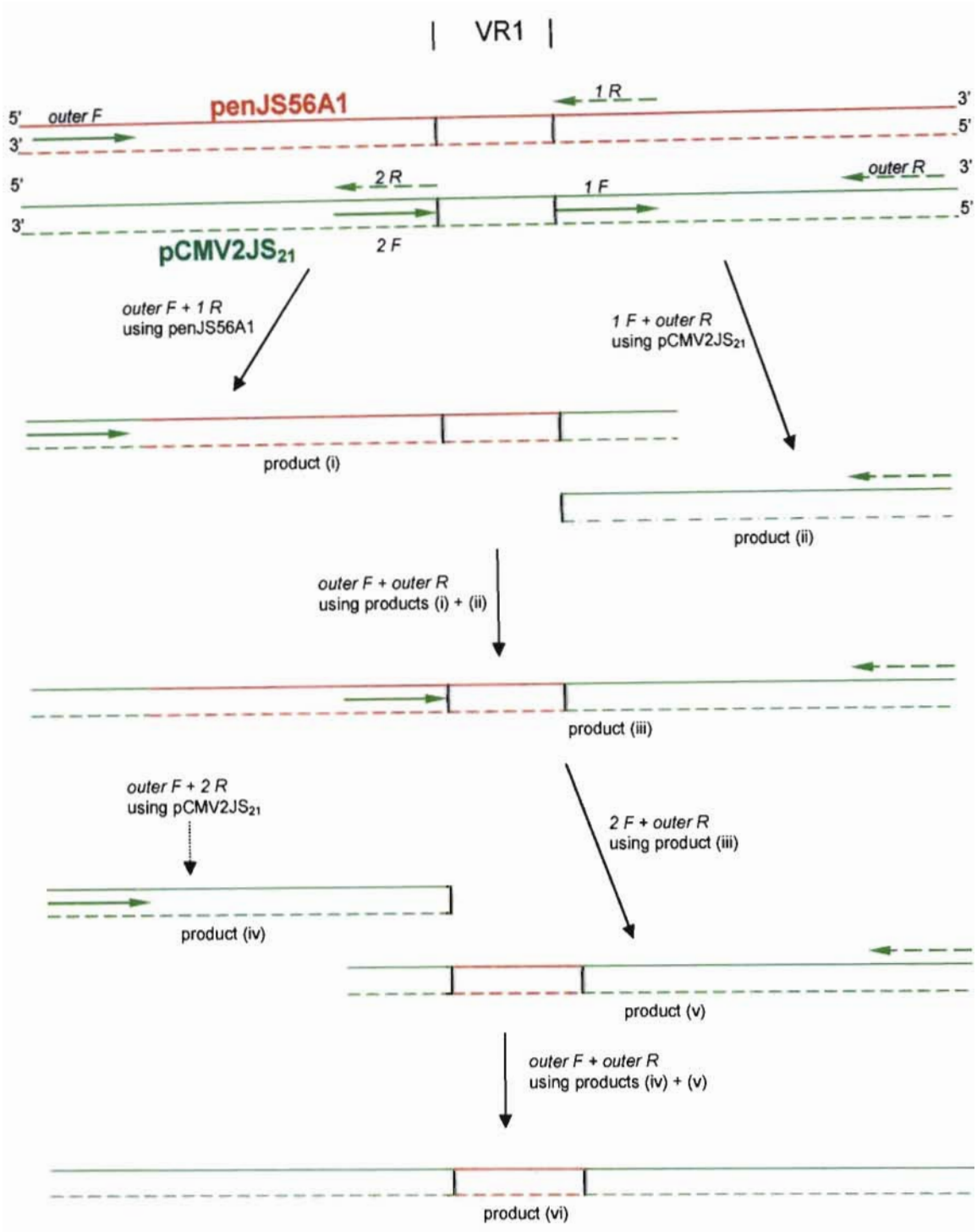



**Figure 3.1 Conceptual framework showing how the sequential use of semi-overlapping PCR products could theoretically be employed to create an amplicon consisting of pCMV2JS$_{21}$-derived sequences (green) flanking the penJS56A1 (red) VR1.** The position of VR1 is indicated at the top of the schematic. Forward and reverse sequences and primers are indicated by solid and dashed lines and arrows, respectively. The green colour of the arrows indicates that the primers are based on the sequence of pCMV2JS$_{21}$. They would be expected to amplify enJS56A1 sequences owing to the high degree of sequence homology of exogenous and endogenous JSRVs outside of the variable regions.

on the high degree of sequence homology between the endogenous and exogenous JSRVs, and all PCR primers are based on the sequence of pCMV2JS$_{21}$. Product (i), for instance, would be generated using pCMV2JS$_{21}$-based primers, and penJS56A1 as a template. This PCR amplicon would be primarily penJS56A1-derived, but with pCMV2JS$_{21}$-derived tails corresponding to the primers used. Product (ii) would be entirely pCMV2JS$_{21}$-based and its sequence would partially overlap with that of product (i) immediately 3' of VR1.

A combination of products (i) and (ii) in a PCR would lead to the extension of each of these products (they would each act initially as both primer and template), forming a new chimeric amplicon, product (iii). In the same PCR, product (iii) would be amplified with the primers indicated in Figure 3.1. Product (iv) would be another entirely pCMV2JS$_{21}$-based amplicon terminating at the 5' junction of VR1. Eventually, continuing to use the primer-based tails as perfectly homologous overlaps between the combined templates, a chimeric product (vi) could be generated that would be pCMV2JS$_{21}$-based, with the exception of the precise substitution of the penJS56A1-derived VR1.

Having developed this basic framework that could theoretically be implemented, it was reasoned that if two semi-overlapping PCR products could be used to produce and subsequently amplify a self-generating (by means of sequence extension by *Taq* DNA polymerase [*Taq*]) combined template, it should also be possible to use three, albeit at the possible forfeiture of some degree of efficiency. The model was therefore revised so as to reduce the number of steps and intermediate products involved. At the same time, candidate primers from existing stocks, as well as potential RE enzymes for use in subsequent steps, were identified (Fig. 3.2). The changes made to the strategy principally entailed the amplification of penJS56A1 VR1 using pCMV2JS$_{21}$-based primers

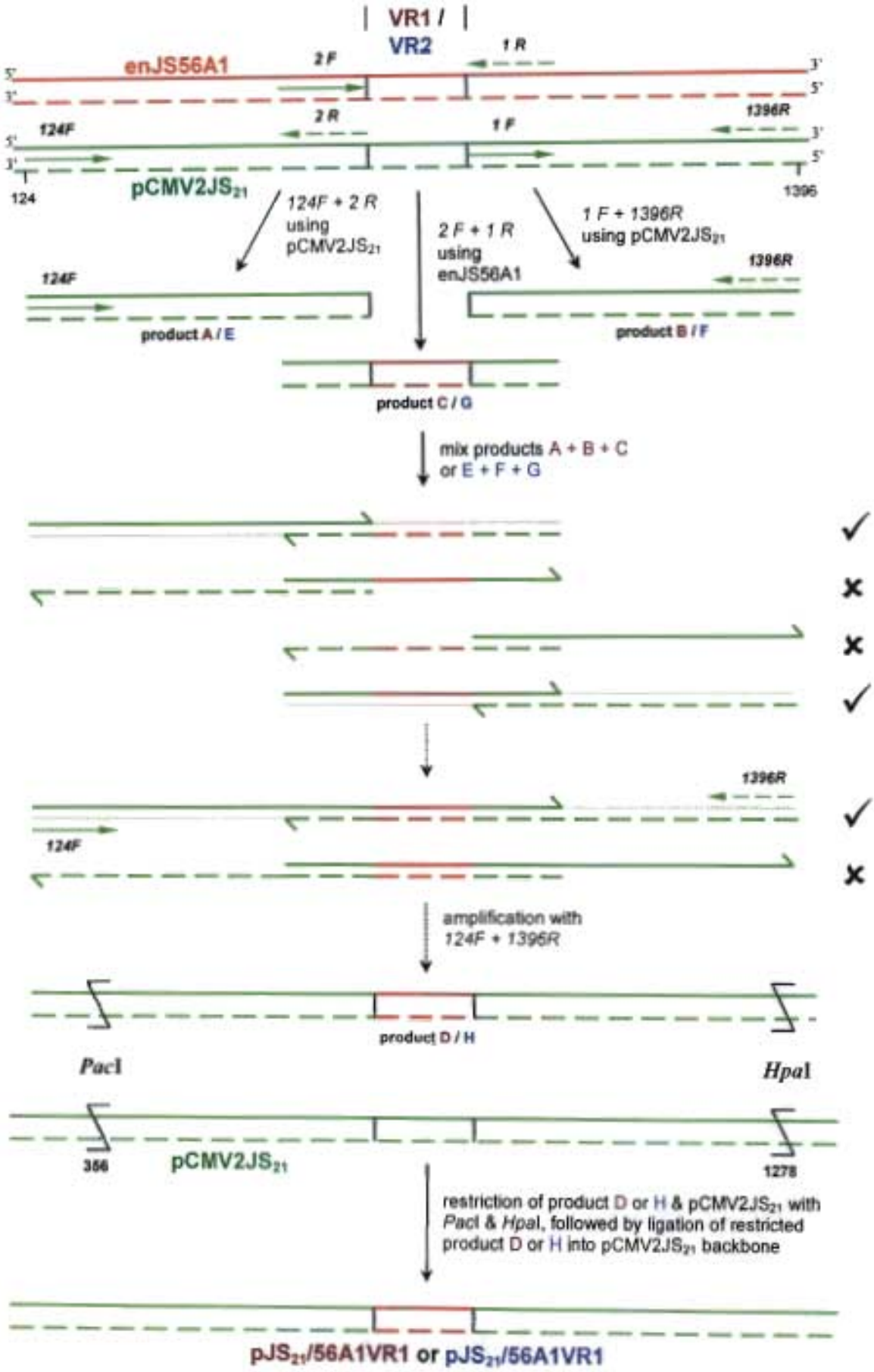

VR1 / VR2
enJS56A1
2 F
1 R
5'
3'
124F
2 R
1 F
1396R
124
pCMV2JS21
1396
124F + 2 R using pCMV2JS21
2 F + 1 R using enJS56A1
1 F + 1396R using pCMV2JS21
product A / E
product B / F
product C / G
mix products A + B + C or E + F + G
amplification with 124F + 1396R
product D / H
PacI
HpaI
386
1278
restriction of product D or H & pCMV2JS21 with PacI & HpaI, followed by ligation of restricted product D or H into pCMV2JS21 backbone
pJS21/56A1VR1 or pJS21/56A1VR1

**Figure 3.2. Schematic showing the entire strategy that could be implemented for the substitution of the penJS56A1-derived VR1 or VR2 into pCMV2JS$_{21}$.** Three semi-overlapping PCR products (A, B and C pertaining to VR1, shown in maroon, or E, F and G pertaining to VR2, shown in blue) would be generated and used, in combination, as a single template. The positions of primers 124F and 1396R (common to the substitution of either VR1 or VR2) are indicated using the nucleotide sequence numbering of pCMV2JS$_{21}$. The primers 1 F, 1 R, 2 F and 2 R had not been designed at this stage, and would be specific for either VR1 or VR2. Dotted lines indicate *Taq*-mediated primer extension. Extension-competent annealing intermediates are shown with ticks to their right; annealing intermediates incapable of extension are indicated by crosses. The positions of the recognition sequences of the REs (common to the substitution of either VR1 or VR2) that were to be used to digest both pCMV2JS$_{21}$ and product D or H, are indicated.

precisely flanking the VR1, yielding product C. Products A and B would be pCMV2JS$_{21}$-derived, with product A terminating at the 5' VR1 junction, and products B starting at the 3' VR1 junction.

Based on the design of the primers, the sequences of these three products would partially overlap in the upstream and downstream regions flanking VR1. The combining of the three products in the context of a PCR would lead to priming of the overlapping parts of the sequences, followed by *Taq*-mediated extension. In the first round of PCR two extension intermediates would be generated from productive base-pairing. There would also be two non-productively annealing intermediates that would not be extended by *Taq*, since *Taq* synthesises in the 5' to 3' direction (Fig. 3.2). In the next round of PCR, the four strands comprising the two extension intermediates (one forward and one reverse strand each) would anneal in their overlapping regions, giving rise to an extension-competent and an extension-incapable intermediate. The primers included in the reaction would then permit the amplification of the former in subsequent rounds of PCR, giving rise to product D, which had a predicted length of 1285 bp.

A list of unique RE recognition sites pertaining to the sequence of $JSRV_{21}$ was generated using BioEdit (Table 3.1). Enzymes that cut two times or more would not be suitable for the subsequent steps. A further requirement was that they should not cut the vector backbone of $pCMV2JS_{21}$, as this would also create more than one RE product. Out of this list, *Pac*I and *Hpa*I were initially chosen for the upstream and downstream restrictions, respectively. Only once these had been selected, the outer primers 124F and 1396R were

**Table 3.1 List of unique restriction endonuclease recognition sites in $JSRV_{21}$.**

| Restriction endonucleases | Recognition sequence | Cleavage site |
|---|---|---|
| *Aat*II | G_ACGT'C | 4819 |
| *Afe*I | AGC'GCT | 5479 |
| *Age*I | A'CCGG_T | 2917 |
| *Apa*I | G_GGCC'C | 1667 |
| *Apa*LI | G'TGCA_C | 4515 |
| *Avr*II | C'CTAG_G | 7235 |
| *Bae*I | ACnnnnGTAyCnnnnnnn_nnnnn' | 3644 |
| *Bae*I | GrTACnnnnGTnnnnnnnnnn_nnnnn' | 3677 |
| *Bam*HI | G'GATC_C | 5269 |
| *Bcl*I | T'GATC_A | 5542 |
| *Bip*I | GC'TnA_GC | 5433 |
| *Bmg*BI | CAC'GTC | 4895 |
| *Bmt*I | G_CTAG'C | 4152 |
| *Bsa*I | GGTCTCn'nnnn_ | 2353 |
| *Bsa*BI | GATnn'nnATC | 4629 |
| *Bse*YI | C'CCAG_C | 5214 |
| *Bsm*I | GAATG_Cn' | 5353 |
| *Bsr*FI | r'CCGG_y | 2917 |
| *Bst*API | GCAn_nnn'nTGC | 5417 |
| *Eco*RI | G'AATT_C | 3028 |
| *Fsp*I | TGC'GCA | 6580 |
| *Hpa*I | GTT'AAC | 1278 |
| *Nco*I | C'CATG_G | 6798 |
| *Nhe*I | G'CTAG_C | 4148 |
| *Nru*I | TCG'CGA | 7075 |
| *Pac*I | TTA_AT'TAA | 356 |
| *Pci*I | A'CATG_T | 5507 |
| *Pfl*MI | CCAn_nnn'nTGG | 1977 |
| *Ppi*I | GAACnnnnnCTCnnnnnnnn_nnnnn' | 6688 |
| *Ppi*I | GAGnnnnnGTTCnnnnnnn_nnnnn' | 6720 |
| *Psh*AI | GACnn'nnGTC | 2036 |
| *Psp*OMI | G'GGCC_C | 1663 |
| *Pst*I | GAACnnnnnnTACnnnnnnn_nnnnn' | 2454 |
| *Pst*I | GTAnnnnnnGTTCnnnnnnn_nnnnn' | 2422 |
| *San*DI | GG'GwC_CC | 6625 |
| *Sex*AI | A'CCwGG_T | 1365 |
| *Stu*I | AGG'CCT | 6484 |
| *Zra*I | GAC'GTC | 4817 |

chosen, since they were positioned on the outsides of the RE recognition sequences, but within reasonably close proximity compared to other available primers, thereby minimising the length of product D, and the likelihood of introducing PCR-generated sequence errors.

The next step in this substitution strategy was the restriction of both product D and pCMV2JS$_{21}$ with *Pac*I and *Hpa*I, followed by agarose gel excision of the relevant fragments (the restricted product D, and the pCMV2JS$_{21}$ backbone minus the fragment between the *Pac*I and *Hpa*I sites). This was to be followed by T4 DNA ligase-mediated ligation of the two fragments, and transformation of competent *E. coli* cells using the ligation product. Screening of transformed colonies was to be performed by direct colony PCR, using primers 2F and 1R (Fig. 3.2) (since enJS56A1 VR1 is 12 bp longer than JSRV$_{21}$ VR1 these could be distinguished on the basis of size) as well as 124F and 1396R (to ensure that the substituted fragment was not truncated). Colonies containing correctly sized inserts would then be further screened by sequencing (after plasmid preparation) to verify that no PCR-generated sequence errors had been introduced.

The same principles from this method would be used to substitute VR2 to create pJS$_{21}$/56A1VR2 (Fig. 3.2). Furthermore, using pJS$_{21}$/56A1VR1 as starting material, the substitution of VR2 could be repeated to generate a chimeric construct containing both the enJS56A1-derived variable regions, which would be named pJS$_{21}$/56A1VR1VR2.

### 3.2.2.2 Implementing the method

PCR primers for the substitution of VR1 and VR2 were designed precisely at the borders of these variable regions, as shown in Figure 3.3. The general format for the setup of PCRs was followed, as outlined in 2.2.6. The specific conditions employed in this part of the study are summarised in Table 3.2.

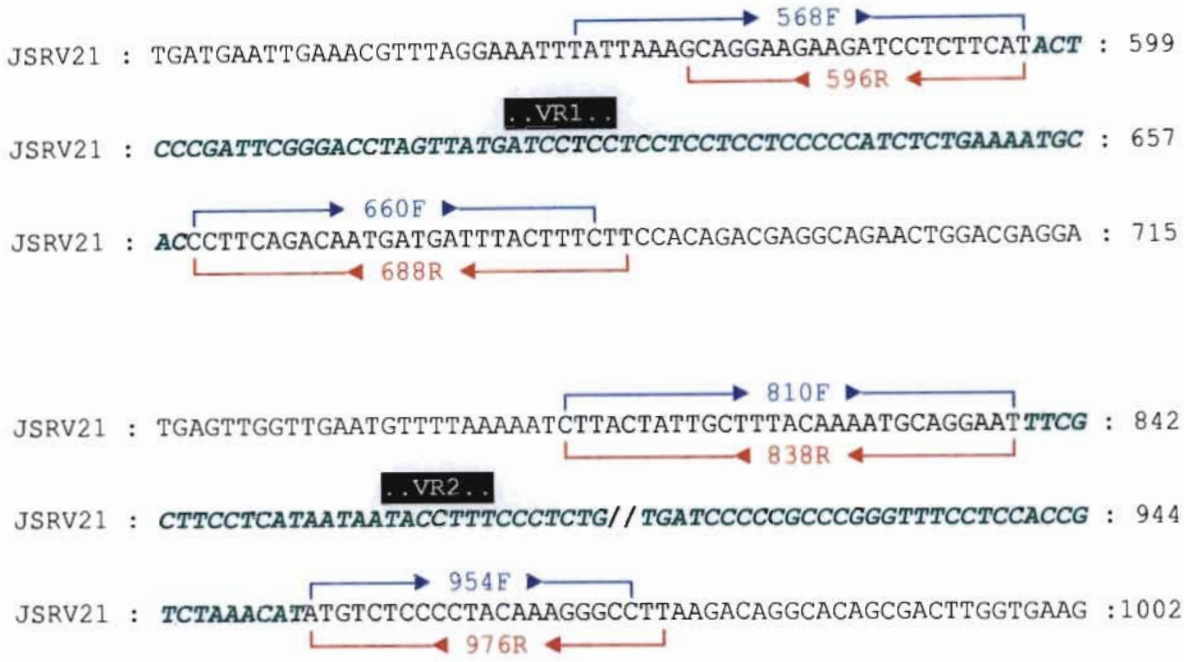



**Figure 3.3 Sequences in $JSRV_{21}$ flanking VR1 and VR2 used to design primers.** The positions of forward primers are shown in blue; those for reverse primers in red. The sequences of VR1 and VR2 are indicated in green italics. Forty-six residues of VR2 have been omitted in this illustration, indicated by the double stroke. Note that priming begins and ends precisely at the borders of VR1 and VR2.

Products A, B and C were created by PCR as shown in Figure 3.2 and Table 3.2. These were mixed 1 µl A + 1 µl B + 2 µl C + 196 µl $dd_iH_2O$ to make a template mix for the PCR generation of product D for the substitution of VR1. Products E, F, G and H were similarly derived for the substitution of VR2 (Fig 3.2 and Table 3.2).

Products D and H, as well as pCMV2JS$_{21}$ (approximately 100 ng each) were double-digested with *Pac*I and *Sex*AI. RE digestion reactions were subsequently combined {D + pCMV2JS$_{21}$} or {H + pCMV2JS$_{21}$} and precipitated with sodium acetate and ethanol, before being reconstituted in the context of T4 DNA ligase reactions. The ligation products were used to transform competent *E. coli* JM105 cells.

**Table 3.2 Details of PCR conditions employed in the creation of VR1- and VR2-substituted constructs.**

| Primer pair | Annealing temper-ature | *Taq* DNA polymerase | Ampli-fication cycles | Template | Product name |
|---|---|---|---|---|---|
| 124F + 596R | 51.8°C | TaKaRa Ex | 35 | pCMV2JS$_{21}$ | A |
| 660F + 1396R | 54.1°C | TaKaRa Ex | 35 | pCMV2JS$_{21}$ | B |
| 660F + 1608R | 51.8°C | TaKaRa Ex | 35 | pCMV2JS$_{21}$ | B (long) |
| 568F + 688R | 52.5°C | TaKaRa Ex / HotStarTaq[1] | 35 | penJS56A1 | C / -- [1] |
| 124F + 838R | 54.2°C | TaKaRa Ex | 35 | pCMV2JS$_{21}$ | E |
| 954F + 1396R | 54.4°C | TaKaRa Ex | 35 | pCMV2JS$_{21}$ | F |
| 954F + 1608R | 51.4°C | TaKaRa Ex | 35 | pCMV2JS$_{21}$ | F (long) |
| 810F + 976R | 56.5°C | TaKaRa Ex | 35 | penJS56A1 | G |
| 124F + 1396R | 55.0°C | TaKaRa Ex / HotStarTaq[1] | 30 | Mix of products A+B+C or E+F+G | D or H / -- [1] |
| 124F + 1608R | 51.7°C | TaKaRa Ex | 30 | Mix of products A+B+C or E+F+G | D (long) or H (long) |
| 481F + 1038R | 53.4°C | HotStarTaq[2] | 35 | Chimeric particle RNA | -- |

[1] When used for screening

[2] Enzyme included in the One-Step RT-PCR kit (Qiagen) for amplification, subsequent to cDNA synthesis by reverse transcription.

For the substitution of VR1, transformants were screened in two stages of direct colony PCR. The first stage employed primers 568F + 688R. Colonies supporting the amplification of a longer product (endogenous VR1 is 12 bp longer than exogenous VR1)

were further screened for full-length inserts using primers 124F + 1396R. For the substitution of VR2, colonies were first screened for full-length inserts by direct colony PCR, using primers 124F + 1396R. Correctly sized PCR products were purified using the High Pure PCR Product Purification Kit (Roche) and RE digested with *Sma*I, since $JSRV_{21}$ VR2 contains a *Sma*I site, whereas enJS56A1 VR2 does not.

Colonies identified as putatively containing constructs with the desired substitutions were expanded in broth culture and their plasmid DNA isolated by MiniPrep. The entire regions substituted by digestion with *Pac*I and *Sex*AI were sequenced using primers 124F, 1396R, and other primers internal to these, to select clones that did not contain any PCR-generated mutations.

The construction of the double recombinant $pJS_{21}$/56A1VR1VR2 constituted an exact repetition of the steps involved in substituting enJS56A1 VR2 into the pCMV2$JS_{21}$backbone, except that $pJS_{21}$/56A1VR1 was used as the backbone into which the substitution of VR2 was made. The screening of transformed colonies was also the same as for the VR2 substitution described above.

### 3.2.3 *In vitro* characterisation of chimeric clones

#### 3.2.3.1 Particle formation assays

The ability of the chimeric clones to support particle formation was assessed *in vitro*. Human 293T cells were transfected at 50% confluence with each of the chimeric clones, as

well as pCMV2JS$_{21}$ as a positive control, using the CalPhos Mammalian Transfection Kit (Clontech). After the initial change of culture medium (16 hours post-transfection), three sets of culture medium were collected from each transfection at 24 hour intervals, and pooled. Viral particles were pelleted by ultracentrifugation over a double glycerol cushion. Pellets of viral particles were reconstituted in TNE buffer and used as samples in Western blots. Particle formation was assayed by the detection of JSRV p26 capsid protein.

#### 3.2.3.2 Confirmation of particle formation specificity

In order to confirm that the retroviral particles detected by Western blotting had originated specifically from the respective chimeric constructs used to transfect 293T cells, RNA was extracted from concentrated retroviral particles (see 3.2.1.10). The RNA (6 µl of 66 µl) was used as a template in a OneStep RT-PCR (Qiagen) amplification according to the manufacturer's instructions, employing a JSRV$_{21}$ sequence-specific downstream primer 1038R for cDNA synthesis, and the same primer in combination with an upstream primer 481F (see Appendix II for primer sequences and Appendix III for primer annealing positions) for PCR amplification of a region spanning both VR1 and VR2. The final concentration of each primer was 0.6 µM. The reactions were carried out in a Biometra TGRADIENT thermocycler using the following cycling conditions: 50°C for 30 min; 95°C for 15 min; 35 cycles of 94°C for 35 s, 53.4°C for 1 min, and 72°C for 1 min; 72°C extension for 5 min. For negative (no reverse transcription) controls, HotStarTaq (Qiagen) was used in the absence of the reverse transcriptases Omniscript and Sensiscript contained in the OneStep RT-PCR Kit. The resulting RT-PCR products were purified from an

agarose gel using a QIAquick Spin Gel Extraction Kit (Qiagen) and subsequently sequenced using the same primers (481F and 1038R).

#### 3.2.3.3 Infectivity assays

Sheep choroid plexus cells were infected with chimeric retroviral particles as described in Section 3.2.1.11. Infected cells were maintained in culture in the presence of Polybrene. At various passages post-infection, genomic DNA was isolated from infected cells using a DNeasy Tissue Kit for Cultured Cells (Qiagen). The presence of integrated JSRV provirus was determined by use of an exogenous-specific JSRV U3 PCR (Palmarini *et al.*, 1996b), utilising primers P-I and P-III (see Appendix III for primer annealing positions in proviral JSRV), adapted by using HotStarTaq DNA polymerase (Qiagen).

## 3.3 RESULTS

### 3.3.1 Substitution of VR1 and VR2

A complex strategy was designed that facilitated the precise substitution of the enJS56A1-derived VR1 and VR2 (both individually and in combination) into the infectious molecular clone pCMV2JS$_{21}$. The precision of this novel method is emphasised because an alternative and more conventional approach to make these substitutions would have been to identify RE sites on either side of VR1 (and separately, VR2) that are shared by enJS56A1 and JSRV$_{21}$. If no shared RE sites could be identified, they could have been created by site-directed mutagenesis in such a way that the amino acid sequence of the resulting constructs would remain unchanged (*i.e.* silent mutations). Clones of both these sequences (penJS56A1 and pCMV2JS$_{21}$) could then have been digested with the two REs, and the fragment containing enJS56A1 VR1 could have been ligated into the backbone of pCMV2JS$_{21}$. The problem with this approach would have been that any single-nucleotide differences between enJS56A1 and pCMV2JS$_{21}$ located in close proximity of VR1 would also have been substituted, thereby confounding any findings down the line. If functional differences are identified between VR1 of enJS56A1 and JSRV$_{21}$, could they be categorically attributed to VR1 if there are other (apparently minor) differences outside VR1 in the substituted region?

In this study, three chimeric constructs were created, each based on the backbone of pCMV2JS$_{21}$, but containing the VR1, VR2 and both VR1 and VR2 of enJS56A1. The substitutions were made exactly at the boundaries of the variable regions, and the strategy was published (Hallwirth *et al.*, 2005) in its final form (Fig. 3.4).

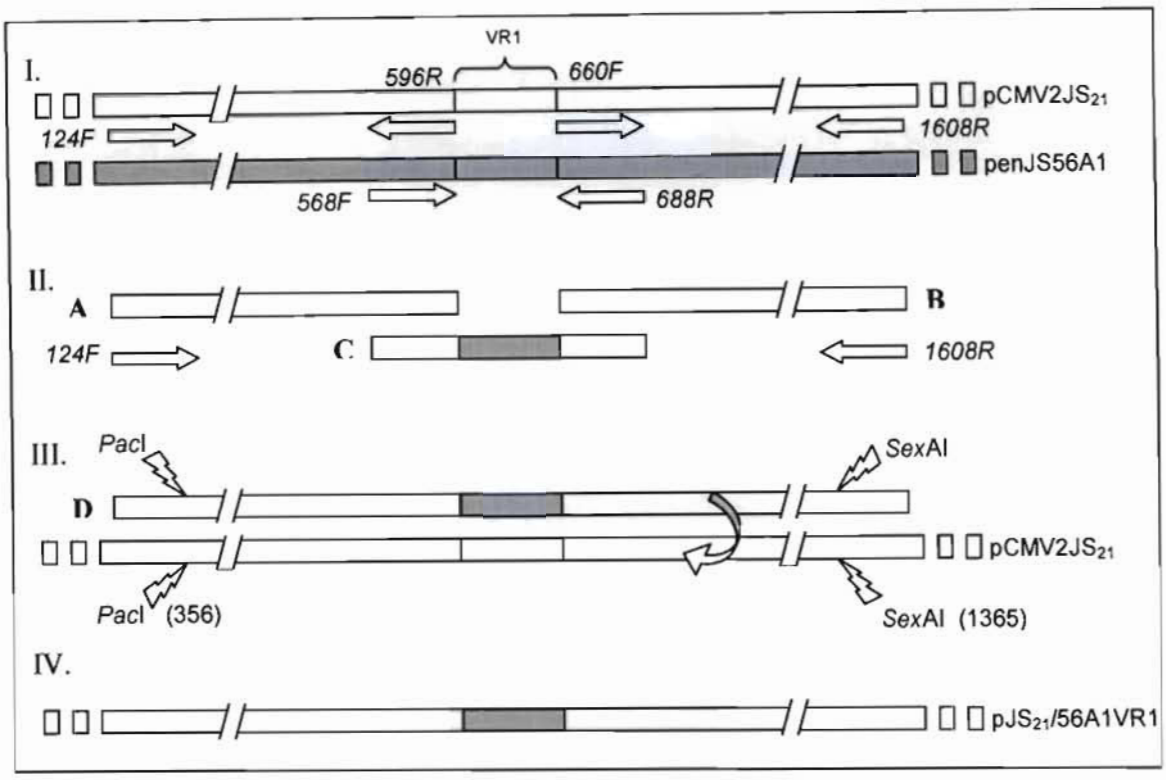



**Figure 3.4 Schematic for the substitution of enJS56A1 VR1 into wild-type JSRV Gag.** (I) The two starting plasmids pCMV2JS$_{21}$ and penJS56A1 are shown. The small vertical rectangles at each side indicate the continuation of the plasmid DNA sequences. The numbering of PCR primers is based on the sequence of JSRV$_{21}$, with arrows indicating the direction of priming. (II) PCR products (A, B and C) resulting from amplification of the three primer pairs in I are shown. The PCR primers used to amplify these three products into a single PCR product (124F and 1608R) are also shown. (III) The resulting PCR product (D) is shown, along with the restriction endonuclease sites (and their positions in JSRV$_{21}$) used for directional cloning into pCMV2JS$_{21}$ backbone. (IV) The resulting chimeric plasmid pJS$_{21}$/56A1VR1 is shown. (Adapted from Hallwirth *et al.* (2005).)(Hallwirth *et al.*, 2005)

### 3.3.1.I Creation of relevant PCR products

Products A and C were generated with the primers indicated in Figures 3.2 and 3.3. Product B was initially generated using the primer pair 660F (as shown) and 1396R. At a later point, the reverse primer 1396R was replaced with 1608R, as indicated in Figure 3.4. All three reactions yielded products of the expected sizes (Fig. 3.5, left panel). The balance of the products was run on another agarose gel for excision and gel-purification of the

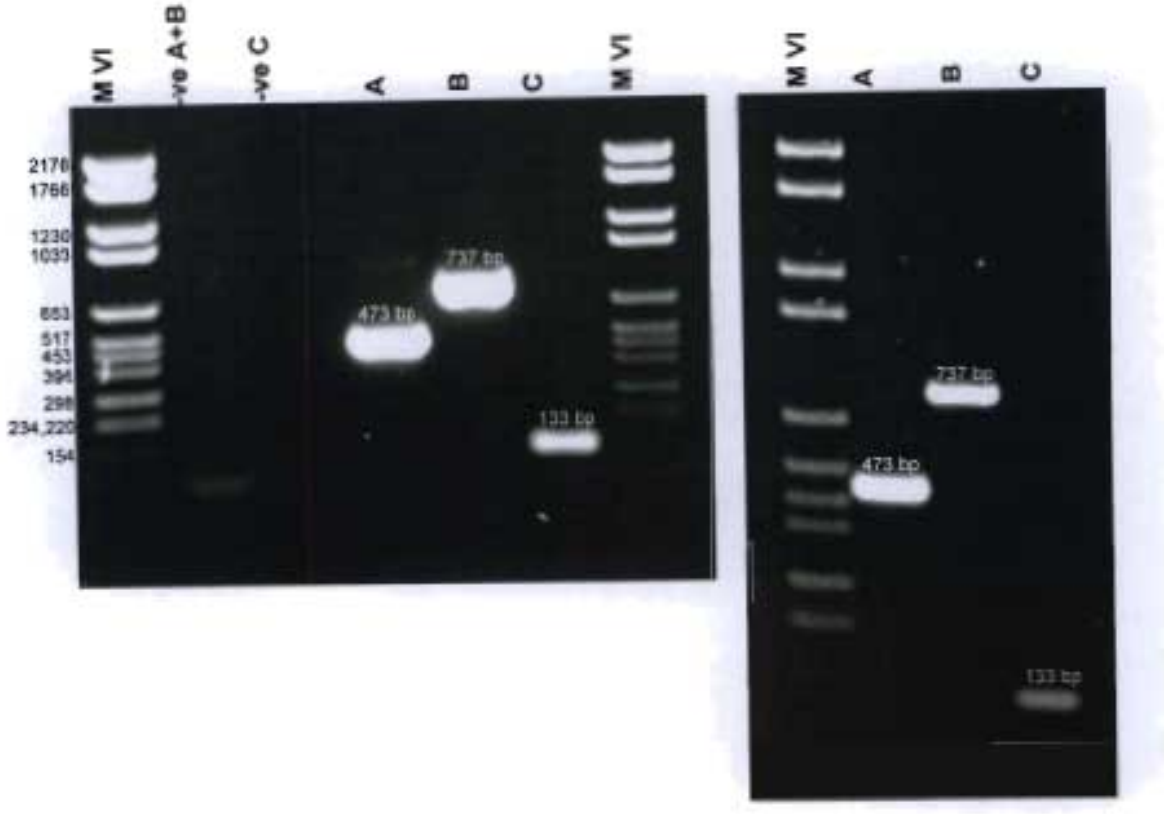



**Figure 3.5 Agarose gels of products A, B and C used in the substitution of VR1.** Left panel: PCR products; right panel: PCR products from left panel, after agarose gel extraction.

bands, using a QIAquick Spin Gel Extraction Kit (Qiagen). Aliquots (10% by volume) of the purified eluates were visualized on a further agarose gel (Fig. 3.5, right panel) to ensure that the extractions had been successful.

For the generation of product D, gel-purified products A, B and C were included in the PCR as a combined template, which was amplified initially using primers 124F and 1396R (Fig. 3.2), and later with 124F and 1608R (Fig. 3.4). pCMV2JS$_{21}$ and penJS56A1 were used as positive control templates. The generation of product D was successful on the first attempt, and the balance of the product was resolved on another gel for excision and

purification. The gel purification was of greater importance at this stage than it had been for products A, B and C, since shorter amplification products had also been generated in the PCR (Fig. 3.6, left panel). An aliquot (2% by volume) of the gel extraction eluate was visualised on an agarose gel to ensure that the purification had been successful (Fig. 3.6, right panel).

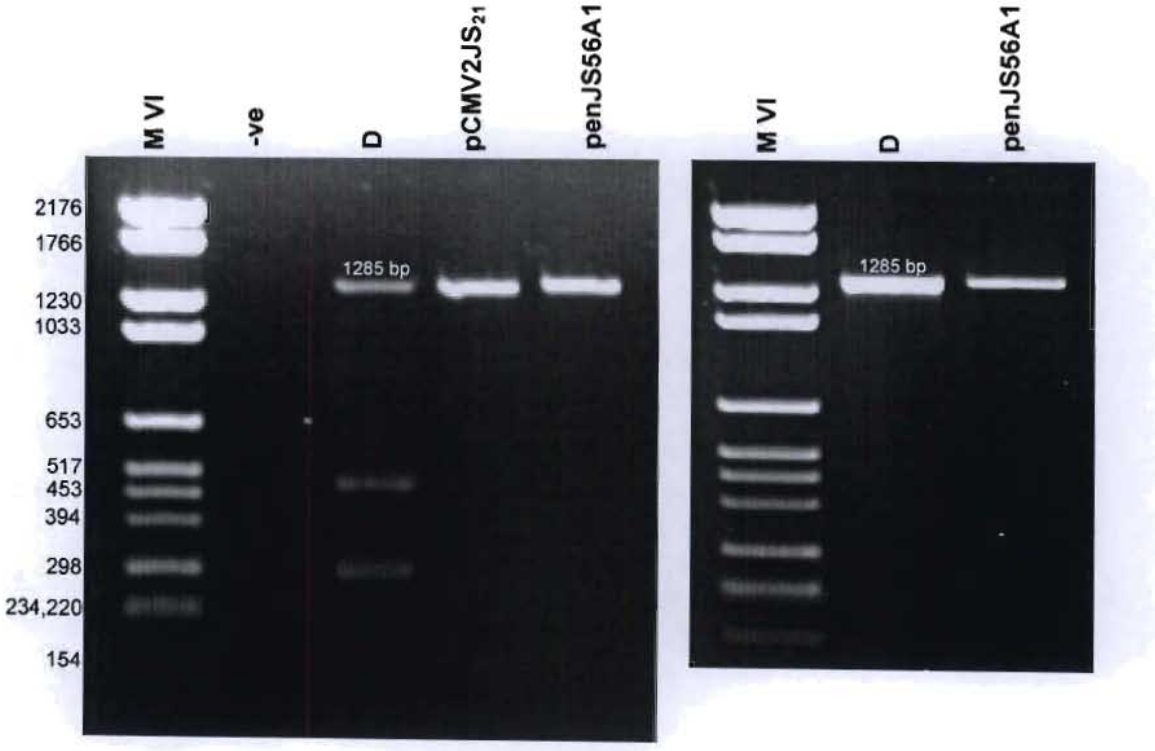



**Figure 3.6 Agarose gels showing the VR1-substituted product D that was generated from a combined template of products A, B and C.** Left panel: PCR product D, and two positive controls where exogenous and endogenous JSRV plasmids had been used to compare the size of product D. Right panel: Gel-extracted product D and PCR product generated with the same primers from penJS56A1.

Essentially the same approach was followed for the PCR-based steps of the substitution of enJS56A1 VR2 into pCMV2JS$_{21}$. Primer pair 124F and 838R was used to generate the JSRV$_{21}$-based product E, terminating at the 5' junction of VR2. Primers 954F and 1608R (initially 1396R) were used to generate the JSRV$_{21}$-based product F, beginning at the 3' junction of VR2. Product G was an enJS56A1-based product flanked by JSRV$_{21}$

sequences, 29 bp on the 5' end and 23 bp on the 3' end, corresponding to the lengths of the $JSRV_{21}$-based primers used, *viz.* 810F and 976R (Fig 3.3). The semi-overlapping products E, F and G were mixed to form a combined template, and amplified using primers 124F and 1608R (initially 1396R). This yielded a $JSRV_{21}$-based product H wherein the VR2 was derived from enJS56A1 (data not shown).

### 3.3.1.2 Endonuclease restrictions and ligations to reconstitute chimeric constructs

In order to bring product D, containing the enJS56A1-derived VR1, into the $pCMV2JS_{21}$ backbone to create the chimeric construct $pJS_{21}$/56A1VR1, both product D and $pCMV2JS_{21}$ needed to be digested with unique RE that cut $pCMV2JS_{21}$ only in one position each. The initial choice for these restrictions was *Pac*I (New England Biolabs) on the 5' side and *Hpa*I (Roche) on the 3' side (Figures 3.2 and 3.7).

Upon repeated failure of subsequent ligation and / or cloning steps, the 3' ligations were carried out with *Sex*AI (New England Biolabs) in place of *Hpa*I, hoping that the sticky ends generated by *Sex*AI would be more amenable to ligation than the blunt ends generated by *Hpa*I. At the same time, primer 1396R was replaced in all reactions with 1608R. This was done for two reasons: Our laboratory stocks of 1396R (which had been brought from CRI/UCI) had been all but depleted, and it was preferable to be able to visualise the "dropped ends" generated by double digestions of products D and H, as seen in Figure 3.7. This was not conveniently possible on the gels being used to visualise the success of the double digestions when product D, generated using the downstream primer 1396R, was

digested with *Sex*AI, as the predicted size of the 3' "dropped fragment" was only around 30 bp.

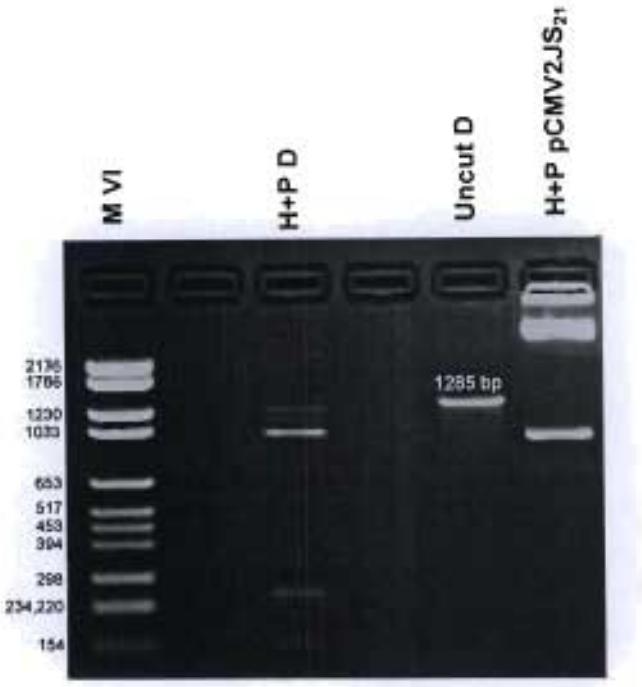



**Figure 3.7 Agarose gel showing the RE digestion products of pCMV2JS$_{21}$ and product D.** DNA was double-digested with *Hpa*I ("H") and *Pac*I ("P"). The fainter bands in the double-digested product D lane represent partial digestion products, and sequences cleaved off either end of product D. The band that was of the same size as the fragment dropped from the double digestion of the vector was gel excised, as was the vector backbone (upper band in the far right lane).

The RE activity of *Sex*AI is blocked by *dcm* methylation. Consequently, the use of *Sex*AI required unmethylated pCMV2JS$_{21}$ DNA. This was obtained by making *E. coli* K12 GM2163 cells (New England Biolabs), a Dam$^-$ and Dcm$^-$ strain, competent by the calcium chloride method, and transforming them with pCMV2JS$_{21}$.

When attempts at ligating and cloning gel-excised fragments failed, using either 124F and 1396R-generated product D digested on the 3' end by *Hpa*I, or 124F and 1608R-generated product D digested on the 3' end by *Sex*AI, a "dirty ligation" strategy was adopted. In place of excising the specific DNA bands of interest after RE digestion with *Pac*I and

*Sex*AI prior to ligation, the DNA of each of the double digestion products was precipitated with sodium acetate and ethanol and reconstituted in $dd_iH_2O$. A large molar excess of the product D double digestion relative to the $pCMV2JS_{21}$ double digestion was included in the subsequent ligation step. This approach had an obvious drawback in that it was expected to result in some degree of reconstitution of $pCMV2JS_{21}$, since the fragment dropped by *Pac*I and *Sex*AI digestion of the plasmid would still be included in the ligation mix. Also, if the RE digestion of the plasmid had not gone to completion in the first place, there would still be intact $pCMV2JS_{21}$ left. It was therefore anticipated that a large number of transformed colonies would need to be screened in order to identify those containing the substituted VR1.

### 3.3.1.3 Screening of transformants

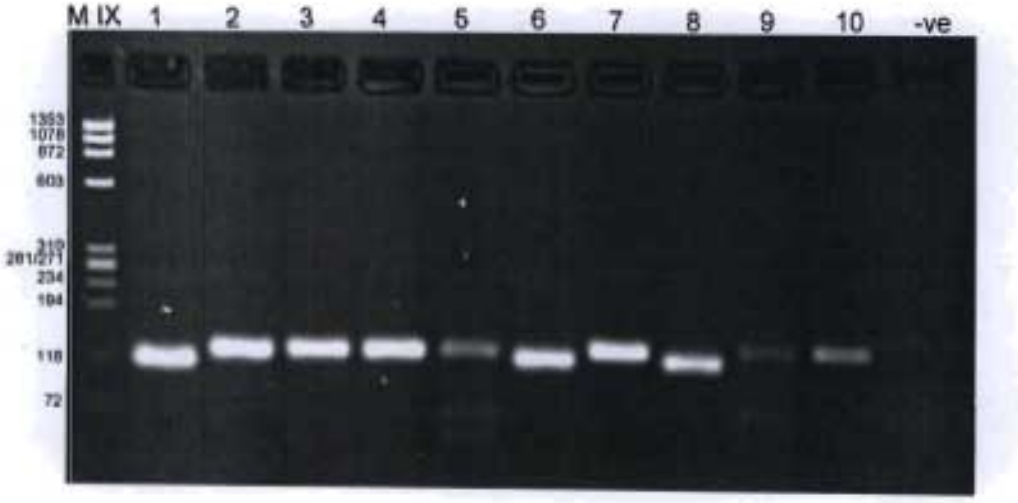



**Figure 3.8 First stage of screening VR1-substituted transformant colonies.** Agarose gel showing the results of direct colony PCRs of *E. coli* colonies generated by transformation with the ligation products of double-digested $pCMV2JS_{21}$ and product D. Endogenous VR1 is 12 bp longer than its exogenous counterpart, so the lanes with larger fragments (2, 3, 4, 5, 7, 9 and 10) represent putative VR1-substituted colonies.

Screening of the putative VR1-substituted transformants was performed in two rounds of direct colony PCR. The first used the primer pair 568F and 688R that had been employed to amplify VR1 to discern the 12 bp size difference between the endogenous and exogenous VR1 (Fig. 3.8). Colonies containing the longer fragment were further screened with primers 124F and 1396R to eliminate truncated products that also arise from the substitution strategy employed (Fig. 3.4). Colony #1 was included in the experiment shown in Figure 3.9 as a control because it was expected to be $JSRV_{21}$-derived, based on the results shown in Figure 3.8, and thus full-length.

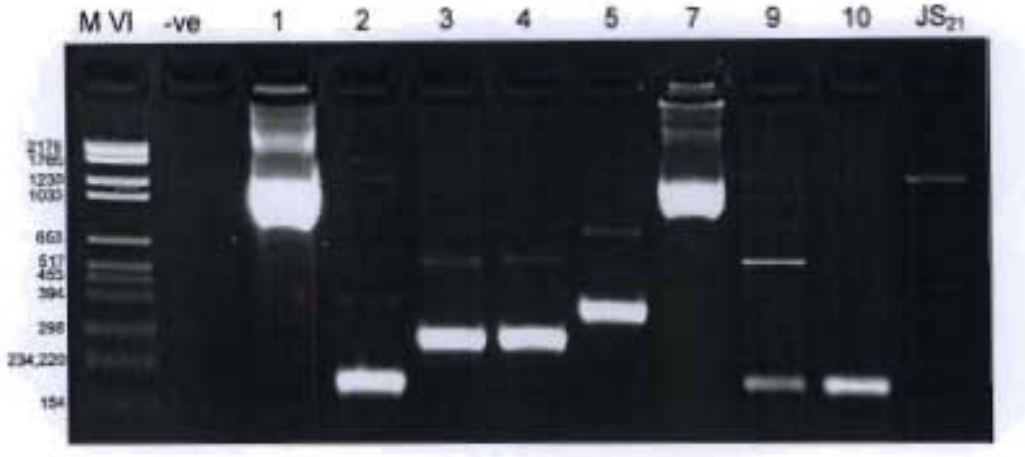



**Figure 3.9 Second stage of screening VR1-substituted transformant colonies.** DNA from colonies that yielded larger fragments in the first round of screening was used in a second round of direct colony PCR to identify colonies containing full-length inserts for the substituted fragment. Based on the previous round of screening, colony #1 was assumed to be of exogenous origin, and therefore full-length (since the fragment inserted into the vector backbone would not have been derived from the mixed template comprising products A, B and C). It was included as a positive size control in this experiment. Only colony #7 conformed to this size out of the colonies shown in this agarose gel.

Colony #7 was the only one in Figure 3.9 that yielded a full-length product. This, together with colonies #13, #15 and #16 (which yielded the same results to this stage; data not shown) were expanded and their plasmid DNAs extracted by MiniPrep for the purpose of sequencing the entire region substituted during the ligation step, using primers 124F, 542F, 976R and 1396R.

The sequenced regions of clones #15 & #16 were identical, containing the same two errors relative to the predicted sequence – one transition (A to G) and one transversion (A to T). Clone #13 had four transitions (G to A; C to T; T to C and A to G). Clone #7 had only a single transition (A to G; Fig. 3.10), which was later corrected (see Section 3.3.1.4).

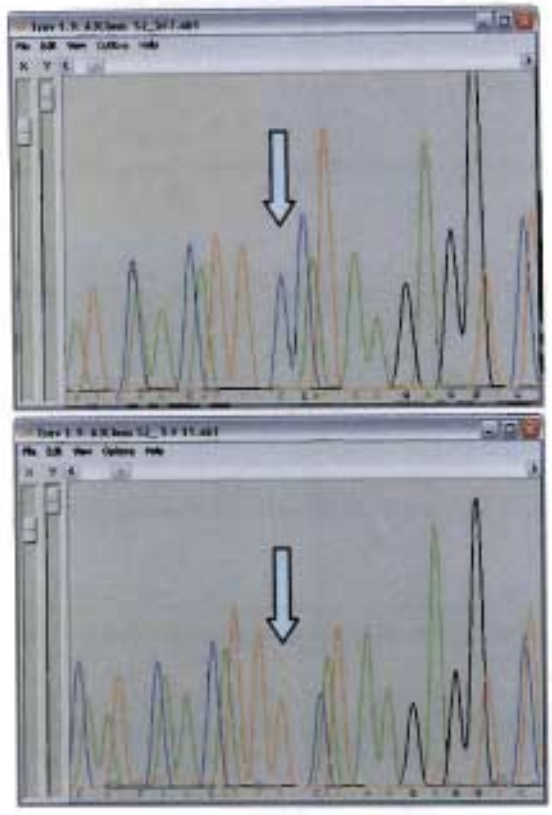


**Figure 3.10 Single point mutation in VR1-substituted clone #7.** The transition mutation is indicated in the top panel by a light blue arrow. The wild-type equivalent is shown in the bottom panel. The sequences shown are reverse sequences, thus the transition that appears as T to C in this figure represents an A to G transition in the forward direction.

VR2s derived from enJS56A1 and $JSRV_{21}$ differ in length by only three base pairs. This difference could not be resolved electrophoretically (data not shown), so a different screening strategy was employed. For the VR2 substitution, colonies were first screened for full-length inserts by direct colony PCR using primers 124F and 1396R, as in the second step of screening above. Correctly sized PCR products were then digested with RE

*Sma*I (Roche Applied Science) to distinguish the endogenous from the exogenous VR2, since $JSRV_{21}$ VR2 contains a *Sma*I site, whereas enJS56A1 VR2 does not (Fig. 3.11).

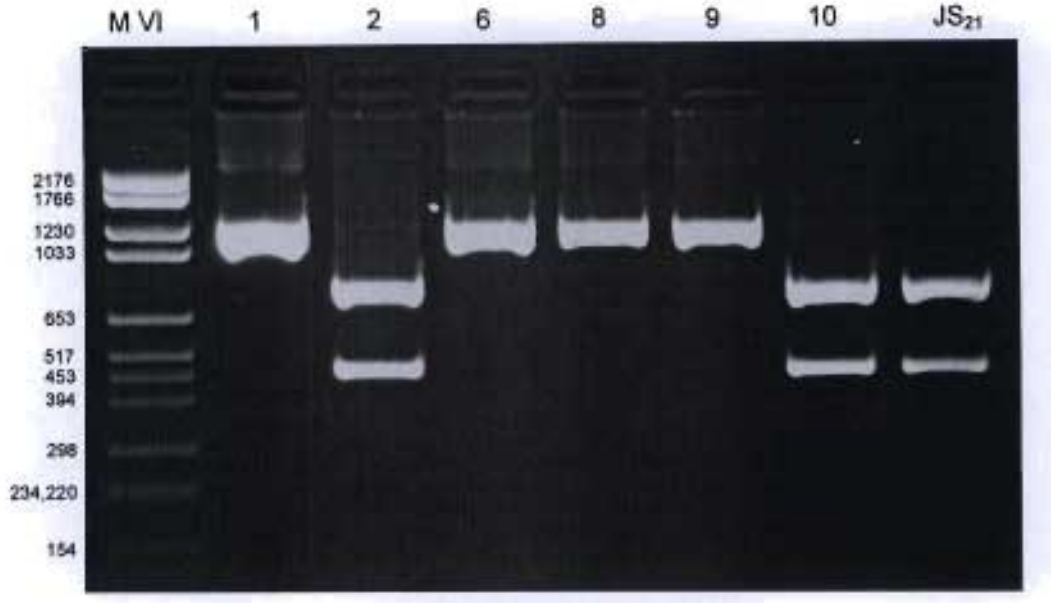



**Figure 3.11 Second stage of screening VR2-substituted transformant colonies.** The first stage of screening was conducted in the same manner as the second stage of screening VR1-substituted transformants. The PCR products derived from amplification with primers124F and 1396R were RE digested with *Sma*I to distinguish endogenous from exogenous VR2. enJS56A1 lacks a *Sma*I recognition site, whereas JSRV21 VR2 contains one. This agarose gel identified colonies 1, 6, 8 and 9 as putatively containing the correct VR2 substitution.

Plasmid DNA was prepared from colonies #1, #6, #8 and #9 by MiniPrep, and the region of interest sequenced in clones #1 and #8 as for the VR1-substituted clones. Whereas clone #8 contained one transition and one transversion point mutation, the sequence of clone #1 matched that of a predicted VR2-substituted fragment in all positions, and no further screening was required. The first chimeric clone to have been successfully generated was therefore $pJS_{21}$/56A1VR2.

### 3.3.1.4 Correcting the mutation in $pJS_{21}$/56A1VR1

In place of correcting the point mutation in the VR1-substituted clone #7, one could have continued to screen more transformed colonies. Since all 20 available colonies had already been screened, this could only have been done by repeating the steps employed in the generation of these clones. Regrettably, the box containing the concentrated stocks of the four PCR primers specific to the generation of the VR1 substitution had been rendered untraceable pursuant to a laboratory freezer failure and the temporary relocation of the freezer's contents to other freezers. The aliquots of working dilutions of the primers would have sufficed to generate products A, B and C, but no subsequent screening of clones (as shown in Fig. 3.8) would have been possible. Before ordering more primers, the author decided to attempt a different approach to correct the point mutation. Since it was positioned downstream of the *Hpa*I site, this RE could be used in combination with another RE that only cuts once in $pCMV2JS_{21}$, downstream of the mutation, to yield a $JSRV_{21}$-derived product without any mutations. *Xba*I, which had been used to digest sheep genomic DNA during the isolation of the integrated $JSRV_{21}$ locus (Palmarini *et al.*, 1999a), was chosen as the second RE to use. Resulting from the CMV promoter insertion during the construction of $pCMV2JS_{21}$, the 5' *Xba*I site was removed, leaving a single 3' *Xba*I site.

*Xba*I is inhibited by *dam* methylation, so unmethylated clone #7 DNA was obtained by transformation of competent *E. coli* GM2163 cells. Double digestion with *Hpa*I and *Xba*I produced two bands, the sizes of which approximated the predicted fragment sizes of $pCMV2JS_{21}$ digested with these two REs (Fig. 3.12).

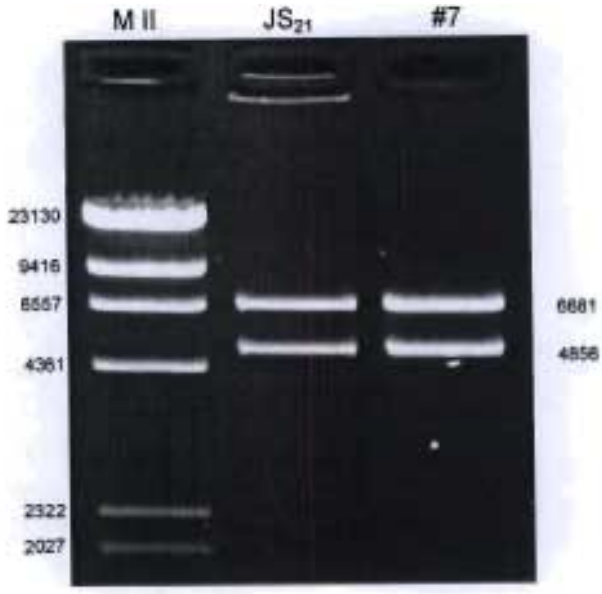



**Figure 3.12 Double digestion of VR1-substituted clone #7 and pCMV2JS$_{21}$ (indicated as JS$_{21}$) with *Hpa*I and *Xba*I.** The predicted sizes of cleavage products are indicated on the right, based on the sequence of pCMV2JS$_{21}$.

The bands were initially gel-purified, and the larger (6681 bp) band derived from pCMV2JS$_{21}$ was ligated to the smaller band (4856 bp, containing enJS56A1 VR1) derived from clone #7. This approach, once again, yielded no transformants, so the two restriction reactions were included in a ligation reaction, and this was used to transform competent *E. coli* JM105. Colonies were tested in duplicate with the exogenous VR1-specific primers 632F and 613F, and 976R as a reverse primer. Those colonies that displayed an absence of exogenous VR1 putatively contained the enJS56A1-derived VR1, and were further screened with primers 124F and 1396R to confirm they contained full-length inserts.

Of the colonies that were confirmed, to this stage, to contain the enJS56A1-derived VR1, four were expanded for MiniPrep plasmid isolation. The next step of screening entailed sequencing with primer 1396R only to identify those clones not containing the point mutation that this strategy had sought to eliminate. Clone #6 was the single one not to contain the mutation. The balance of the region substituted by restriction and ligation

during the construction of clone #7 was sequenced in clone #6 DNA using primers 124F, 542F and 976R, and shown to be correct. The second chimeric clone to be obtained was therefore $pJS_{21}$/56A1VR1.

### 3.3.1.5 Construction of the double-substituted chimera

Having made both $pJS_{21}$/56A1VR1 and $pJS_{21}$/56A1VR2, either of them could have been used as a starting template to additionally substitute the other VR, *i.e.* enJS56A1 VR2 could have been introduced into $pJS_{21}$/56A1VR1, or enJS56A1 VR1 into $pJS_{21}$/56A1VR2. The loss, mentioned above, of the VR1 substitution primers however, dictated that the former approach was pursued. The substitution of enJS56A1 VR2 into $pJS_{21}$/56A1VR1 utilised the same steps as the generation of $pJS_{21}$/56A1VR2. Briefly, product E' (equivalent of product E, last paragraph, Section 3.3.1.1) was created using $pJS_{21}$/56A1VR1 as a PCR template. Products F and G did not need to be created again, and were mixed with product E' to generate product H'. This was digested with *Pac*I and *Sex*AI. The resulting RE digestion products were combined with *Pac*I- and *Sex*AI-digested $pCMV2JS_{21}$ (still available from earlier experiments) in a ligation reaction. This was used to transform competent *E. coli* JM105, and twelve colonies were screened for full-length inserts by direct colony PCR using primers 124F and 1396R. Of the twelve, nine yielded the correctly sized PCR products, which were digested with *Sma*I. Four of those were resistant to *Sma*I digestion, and the corresponding colonies were expanded for plasmid MiniPrep and DNA sequencing. Of the first two clones to be screened by sequencing, one contained only a silent mutation (*i.e.* no change in the predicted amino acid sequence), but it was decided to finish screening the other two available clones. One of them contained no

point mutations. The corresponding colony was expanded, and plasmid DNA prepared by MaxiPrep, to yield pJS$_{21}$/56A1VR1VR2. All three VR-substituted clones had, at this stage, been generated.

### 3.3.2 Particle formation assays

The ability of the JSRV$_{21}$-based chimeric constructs to produce retroviral particles was a requirement for the *in vivo* assays envisaged further down the line, even though such assays were beyond the scope of this particular study. Previous results generated in collaboration with our group (Palmarini *et al.*, 2000b) had localised the defect rendering pCMV2enJS56A1 incapable of releasing particles into the culture supernatant of transiently transfected human 293T cells to the first two thirds of *gag*. In view of the presence of both VR1 and VR2 in this part of the JSRV genome, it was expected that either of these variable regions, or their combined presence, would be responsible for the particle release defect in enJS56A1. The ability of the constructs pJS21/56A1VR1, pJS21/56A1VR2 and pJS21/56A1VR1VR2 to release particles into the supernatant of transiently transfected 293T cells was therefore assayed according to the method of *in vitro* production of JSRV$_{21}$ particles devised by the CRI/UCI (Palmarini *et al.*, 1999a).

The experiment was carried out in triplicate, using plasmid MaxiPrep DNA preparations from three different *E. coli* host strains for each of the constructs, including pCMV2JS$_{21}$ as a positive control: GM2163 (New England Biolabs), JM105 (Amersham Pharmacia Biotech) and DH5α (Invitrogen). Surprisingly, all three chimeric constructs were able to release viral particles into the culture supernatants (Fig. 3.13), and the two constructs

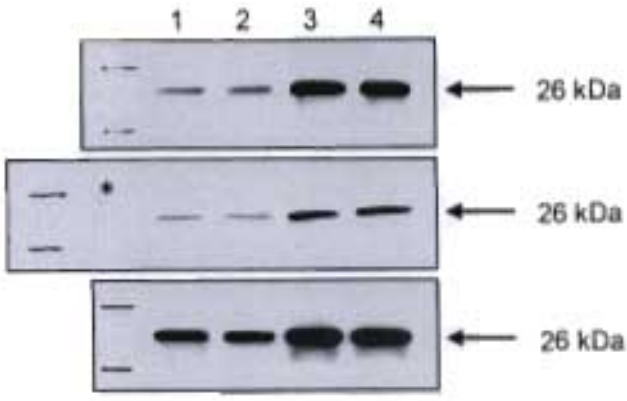



**Figure 3.13 Virus production by VR1 and VR2 chimeras.** Human 293T cells were transiently transfected with pCMV2JS$_{21}$ (lane 1), pJS$_{21}$/56A1VR1 (lane 2), pJS$_{21}$/56A1VR2 (lane 3) and pJS$_{21}$/56A1VR1VR2 (lane 4) derived from transformation of *E. coli* host strains GM2163 (top panel), JM105 (middle panel) and DH5α (bottom panel). The asterisk in the middle panel indicates a mock transfection with pcDNA3.1(-) vector. The 26 kDa JSRV CA antigen was detected using a rabbit polyclonal antibody in a Western blot. All four constructs released virus particles, with those two harbouring the enJS56A1-derived VR2 giving stronger signals. The difference of signal intensity between the three panels relates to the time of exposure of the film to the chemiluminescently labelled membrane.

containing the enJS56A1-derived VR2 consistently gave stronger signals than those expressing the JSRV$_{21}$-derived VR2. Since the ability of all three chimeric constructs to form viral particles was an unexpected result, the initial experiment (Palmarini *et al.*, 2000b) that had led to the anticipation that either VR1 or VR2 (or their combined effect) was responsible for the inability of pCMV2enJS56A1 to support particle release had to be repeated. These results were confirmed by the author to have been correct (Fig. 3.14), in that pCMV2enJS56A1 and pGePEx did not support particle formation, whereas the reciprocal construct of the latter, pGxPEe, did.

The author was made aware of results presented at a conference by another group working on JSRV, which showed that co-transfection of 293T cells with pCMV2JS$_{21}$ and pCMV2enJS56A1 blocked the usually abundant particle release of the former. He was

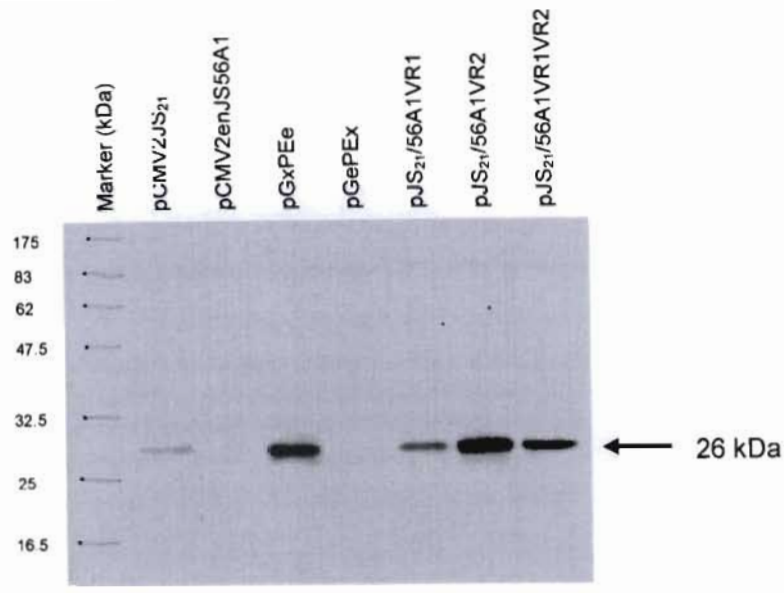



**Figure 3.14 Particle formation assays pertaining to various chimeras of pCMV2JS$_{21}$ and enJS56A1.** The previously published lack of particle release by construct pGePEx containing the first two thirds of enJS56A1-derived *gag* was confirmed by Western blotting.

encouraged to reproduce these results. In addition to that, the author considered it a reasonable assumption that the reported dominant-negative block of JSRV$_{21}$ particle release would be encoded by the first two thirds of enJS56A1 *gag*, since this region also prevents the release of enJS56A1 particles. Furthermore, since the VR1- and VR2-substituted chimeric clones all supported particle release, they could provide the tools to determine whether the dominant-negative block of particle release by enJS56A1 acted specifically on pCMV2JS$_{21}$ VR1 or VR2. Finally, co-transfections were carried out using a 3:1 ratio of pCMV2JS$_{21}$ to either pCMV2enJS56A1 or pGePEx, to determine if the blocking effect was strictly stoichiometric (Fig. 3.15).

The results of the co-transfection experiments showed that the dominant negative blocking effect of particle release was encoded by and confined to some element in the first two thirds of enJS56A1 *gag*, since pGePEx was equally effective at inducing this block as

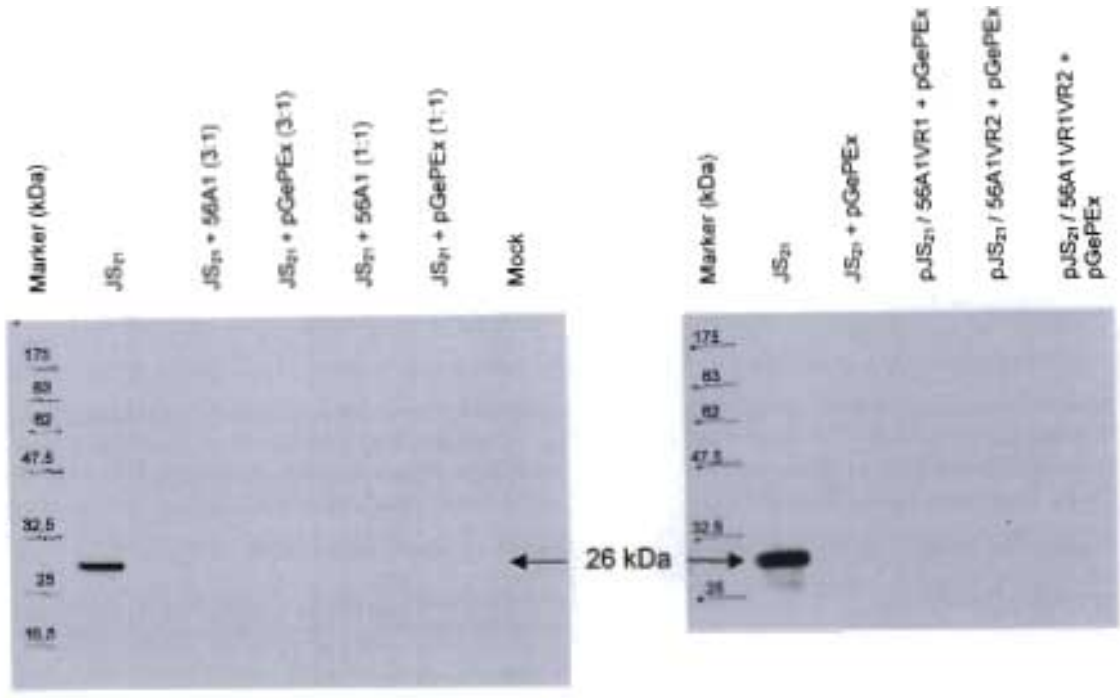



**Figure 3.15 Co-transfection experiments used to narrow down the dominant negative block exerted by enJS56A1 on pCMV2JS$_{21}$-encoded particle release.** JS$_{21}$ = pCMV2JS$_{21}$; 56A1 = pCMV2enJS56A1. All co-transfections of pCMV2JS$_{21}$- with constructs containing at least the first two thirds of enJS56A1 *gag* led to a block of particle release, even when pCMV2JS$_{21}$- was used in three-fold molar excess. Particle release of the VR1- and VR2- substituted clones was equally blocked.

pCMV2enJS56A1 was. The blocking effect could not be overcome by transfecting with a three-fold excess of pCMV2JS$_{21}$ relative to either pGePEx or pCMV2enJS56A1. Finally, pGePEx exerted the blocking effect on the three VR-substituted chimeras in the same manner as it did on pCMV2JS$_{21}$, demonstrating that the presence of enJS56A1-derived VR1 and/or VR2 did not confer any resistance to the blocking mechanism.

### 3.3.3 Proof of particle production specificity

The author maintained that the particles produced from transfection with the chimeric clones ought to be shown to be specifically derived from these constructs, to rule out, for

instance, the remote possibility that the 293T cells had inadvertently been transfected with pCMV2JS$_{21}$. To this end, a method was devised to isolate the genomic RNA of the purified retroviral particles derived from each of the transfections with pCMV2JS$_{21}$, pJS$_{21}$/56A1VR1, pJS$_{21}$/56A1VR2 and pJS$_{21}$/56A1VR1VR2, since no standard protocol or kit was available for this particular application. A primer pair (JS21_481F and JS21_1038R – see Appendix III) was designed that permitted the reverse transcription and

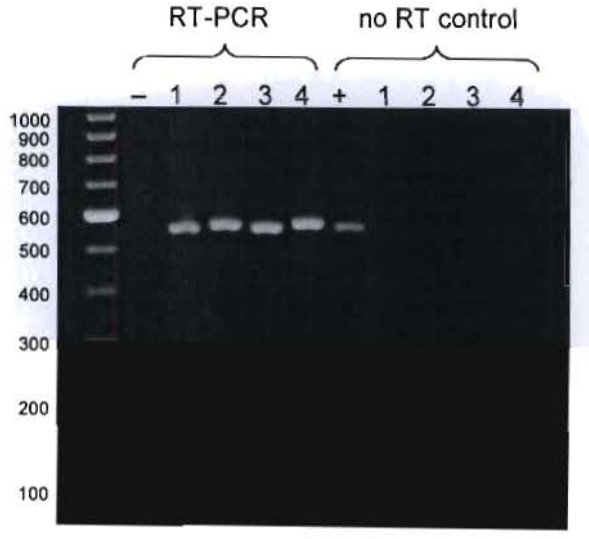



**Figure 3.16 Supernatants from transiently transfected 293T cells were tested for viral RNA by concentration and extraction and RT-PCR for JSRV RNA.** –, no template; +, 5 pg pCMV2JS$_{21}$ DNA (558 bp); 1, pCMV2JS$_{21}$ RNA (558 bp); 2, pJS$_{21}$/56A1VR1 RNA (570 bp); 3, pJS$_{21}$/56A1VR2 RNA (561 bp); 4, pJS$_{21}$/56A1VR1VR2 RNA (573 bp). The size differences of the RT-PCR products arise from the endogenous VR1 and VR2 being 12 and 3 bp longer, respectively, than their exogenous counterparts.

PCR amplification of a part of the JSRV *gag* gene containing both VR1 and VR2. The RT-PCR products (Fig. 3.16) were sequenced, and each of them was found to correspond precisely to the constructs that had been used in the transfections. This constituted definitive proof of the particles' specificity of origin.

### 3.3.4 Chimeric particles are infectious

While the experiments so far showed and independently confirmed the production of virus particles by the chimeric constructs as measured by the release of processed CA antigen, they did not address whether the released virus particles were infectious. To test this, sheep choroid plexus cells were infected with concentrated supernatants from 293T cells transiently transfected with each of the constructs according to a method published by our collaborators at CRI/UCI (Palmarini *et al.*, 1999b). Control infections were carried out with concentrated supernatants that had been incubated at 65°C for 10 min, thereby inactivating retroviral particles contained in the supernatants. This controlled for plasmid

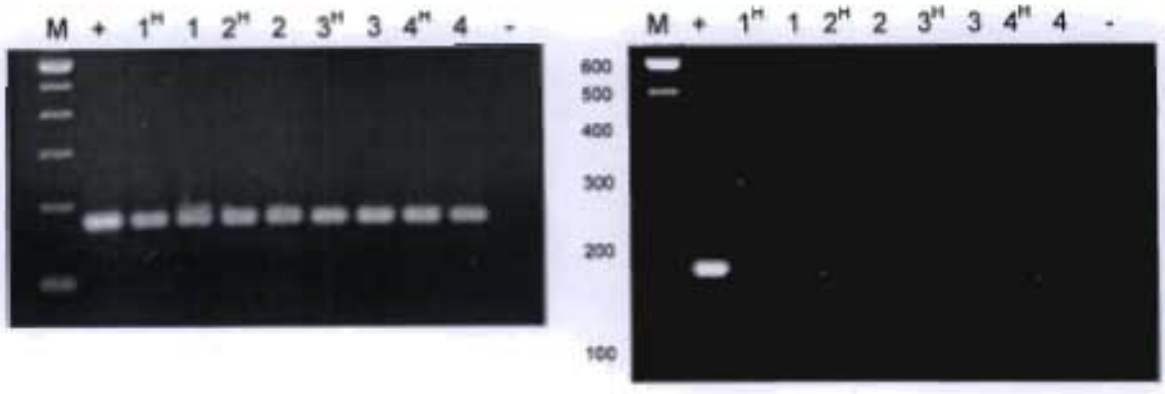



**Figure 3.17 Infectivity assays to determine *in vitro* infectivity of VR1 and VR2 chimeric retroviral particles.** Choroid plexus cell (CP) *in vitro* infectivity assays on supernatants from transiently transfected cells were carried out as described in Section 3.2.3.3. Infected CP cells were tested for the presence of JSRV DNA by JSRV specific PCR followed by agarose gel electrophoresis. Left pane, CP cells at passage 2 post-infection. Right pane, CP cells at passage 5 post-infection. M, 100 bp ladder (Invitrogen); +, 500 ng JS7 DNA; genomic DNA from CP cells infected with particles derived from 1, pCMV2JS$_{21}$; 2, pJS$_{21}$/56A1VR1; 3, pJS$_{21}$/56A1VR2; 4, pJS$_{21}$/56A1VR1VR2; H = heat-inactivated control; –, no template. All constructs produced infectious virus.

carry-over. Genomic DNA was extracted at various passages post-infection. Using an exogenous-specific JSRV U3 PCR (Palmarini *et al.*, 1996b), it was found that all the

particles were infectious as measured by the presence of exogenous viral DNA. DNA from the JS7 lung tumour cell line (Bai *et al.*, 1996; DeMartini *et al.*, 2001), which contains one copy of exogenous JSRV-JS7 per genome, was used as a positive PCR control. As of the fifth passage post-infection, carry-over plasmid DNA was no longer detectable (Fig. 3.17).

## 3.4 DISCUSSION

### 3.4.1 Substitution of VR1 and VR2

#### 3.4.1.1 Creation of VR1 and VR2 substitution chimeras

A conventional RE-based substitution approach was initially recommended to create the VR1- and VR2-substituted chimeras, but the author remained adamant that this would introduce enJS56A1 *gag* point mutations outside of the exact delineation of the variable regions into the chimeras, thereby confounding findings further down the line. Owing to the conceptual complexity of the substitution strategy as it was originally envisaged (Fig. 3.1), its proposed implementation was received with some reservation on the part of the author's peers. The revised strategy admittedly challenged the limits of PCR versatility in that the reaction was expected to create a template from three semi-overlapping PCR products (a function for which PCR is not intended), and amplify this template. This added further to concerns that the approach might not be successful. It was not based on any previously proven methodology that the author or his peers were aware of, but since no other alternative for the precise substitution of VR1 and VR2 had been proposed, it was attempted.

The amplification of products A, B and C comprised standard PCRs, and did not necessitate optimisation. The generation of product D, using products A, B and C as a template mixture, was successful on the first attempt, thereby indicating that the steps comprising the substitution strategy to this point were feasible and translated functionally from theory to practice. The most persistent problems were encountered at the steps where

they were least expected, *viz.* at the stage of ligating the *Pac*I and *Hpa*I (later, *Pac*I and *Sex*AI) double-digested product D into the similarly double-digested backbone of pCMV2JS$_{21}$. Numerous product specialists from different suppliers were consulted on this matter, which led to the attempted use of three different agarose gel extraction kits, different ligase enzymes, and various permutations of reaction conditions. None of these were successful in generating even a single transformant that could be confirmed to contain an insert of the correct size.

The "dirty ligation" approach (see 3.3.1.2), which omitted the gel-purification step after double RE digestion of the vector backbone and the PCR product, was successful on the first attempt, as far as the ligation step was concerned. However, this approach necessitated the design and inclusion of several screening steps that would not have been required if the ligation reaction had comprised only double-digested product D and vector backbone. Nonetheless, the methodology produced the desired clones, which were further verified by DNA sequencing.

#### 3.4.1.2 Broader applications of substitution strategy

Once the problematic aspects had been eliminated from the VR1 substitution strategy, it was relatively simple to re-implement it for the substitution of VR2, and for the introduction of enJS56A1 VR2 into pJS$_{21}$/56A1VR1. The context wherein the strategy was applied relied on the high degree of homology between endogenous and exogenous JSRVs outside of the variable regions that were discovered and defined earlier in this study.

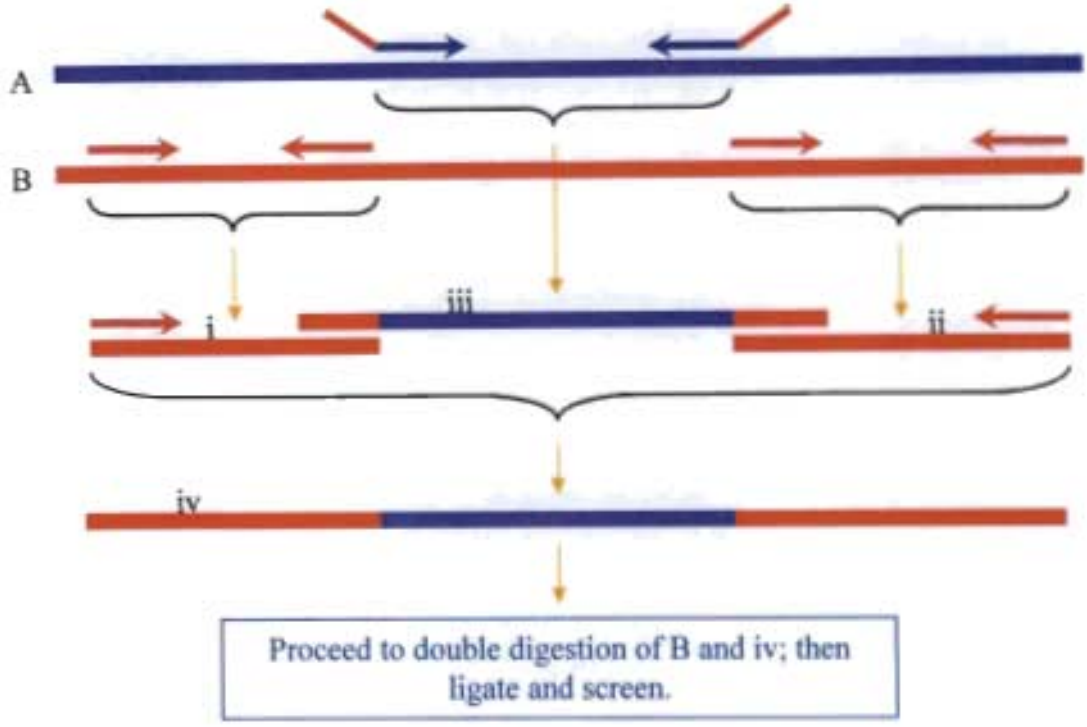



**Figure 3.18 Schematic outlining the application of the substitution strategy developed in this study, adapted to substitute target regions between non-homologous sequences.** The region of interest is amplified from clone A, using A-based primers with tails homologous to the sequences flanking the region to be replaced in clone B, creating product iii. PCR products i and ii also flank the region to be replaced in clone B. Mixing products i, ii and iii in a PCR in the presence of the outer clone B-based primers yields a chimeric product iv. Clone B and product iv can be double-digested with relevant REs, and the restriction products of each ligated to reconstitute clone B, containing the region of interest derived from clone A.

However, the methodology developed here could be applied just as easily to completely unrelated sequences, with suitably designed PCR primers containing "tails", in much the same way that traditional overlap extension PCR is amenable to versatile adaptation [reviewed by Pogulis *et al.* (1996) and Aiyar *et al.* (1996)]. This is demonstrated in Figure 3.18. Note that the size of the substituted regions does not have to be similar; the only requirement is that the tails of the primers used to amplify the region of interest are homologous to the sequences flanking the region to be replaced. This methodology could be applied to introduce PCR-amplifiable genes or genetic elements into any cloned construct.

### 3.4.1.3 Particle formation from chimeric constructs

The particle release defect of enJS56A1 had previously been localised to the first two thirds of *gag* (Palmarini *et al.*, 2000b), where the major differences between endogenous and exogenous JSRV sequences are constituted by VR1 and VR2. It was anticipated that the substitution of the variable regions in *gag* would render one or more of the resulting constructs incapable of releasing viral particles into the supernatant of transfected 293T cells. Upon finding, and independently verifying, that all three chimeric constructs supported particle release, it was concluded that, while the determinants of the particle release defect in enJS56A1 are located in the first two thirds of *gag*, at least some of them (though more likely all) lie outside of VR1 and VR2, despite the high degree of homology between endogenous and exogenous JSRV isolates in these regions.

The two constructs containing the enJS56A1-derived VR2 consistently released more particles into the supernatant of the transfected cells, as measured by Western blot targeting the p26 CA antigen, than did those expressing the $JSRV_{21}$-derived VR2 (Fig 3.13). This interesting observation could not be followed up on by the author, both for lack of financial resources and the limited time period spent at the CRI/UCI. However, given the location of VR2 inside the coding region of *gag* (as opposed to upstream of *gag*), it is not likely that the increase in particle release results from an increase in either transcription or translation. Rather, enJS56A1 VR2-containing particles might be assembled or transported to the cell surface more efficiently, or have an advantage in terms of budding compared to particles containing $JSRV_{21}$-derived VR2.

The unexpected finding that all the VR1- and VR2-substituted chimeras supported particle release into the culture supernatant of transiently transfected 293T cells was presented at one of the Cold Spring Harbor Laboratories' "Retroviruses" meetings, where it stood in contradiction of the results presented by another group. Although the investigators had not focussed on VR2, they had substituted enJS56A1-derived VR1 into the backbone of pCMV2JS$_{21}$, and reported that this construct did not support particle release upon transient transfection of 293T cells. They concluded that VR1 was responsible for the particle

```
other    : GTG GAA ACT TTA TCC TTT TGG ACA TTA ATT CGT GAT TGC CTG GAC : 270
           V   E   T   L   S   F   W   T   L   I   R   D   C   L   D  :  90
56A1VR1  : GTG GAA ACT TTA TCC TTT TGG ACA TTA ATT CGT GAT TGC TTG GAT : 270
           V   E   T   L   S   F   W   T   L   I   R   D   C   L   D  :  90

other    : TTT GAT AAT GAT GAA TTA AAA TGT TTA GGA AAT GTA TTA AAA CAG : 315
           F   D   N   D   E   L   K   C   L   G   N   V   L   K   Q  : 105
56A1VR1  : TTT GAT AAT GAT GAA TTG AAA CGT TTA GGA AAT TTA TTA AAG CAG : 315
           F   D   N   D   E   L   K   R   L   G   N   L   L   K   Q  : 105

other    : GAA GAA GAT CCT CTC CAT GTT CCT GAT TCG GAA CCC AGA TAT GCT : 360
           E   E   D   P   L   H   V   P   D   S   E   P   R   Y   A  : 120
56A1VR1  : GAA GAA GAT CCT CTT CAT GTT CCT GAT TCG GAA CCC AGA TAT GCT : 360
           E   E   D   P   L   H   V   P   D   S   E   P   R   Y   A  : 120

                                   VR1
```

**Figure 3.19 Alignment of nucleotide and amino acid sequences of two VR1-substituted constructs in the part of *gag* upstream of VR1.** 56A1VR1 denotes the construct pJS$_{21}$/56A1VR1 made in this study. The other sequence was theoretically derived from the mention of unpublished results in a review (Palmarini *et al.*, 2004), wherein the substitution of VR1 was from amino acid position 89 to 142 in Gag. enJS56A1-derived sequences are in blue. The seven single nucleotide differences between the two constructs are underlined, and the two resulting amino acid differences are indicated in bold as well. The beginning of VR1 is shown.

release defect of enJS56A1 and probably of enJSRVs in general, since the endogenous form of VR1 was strongly conserved amongst the known enJSRVs. In view of the particle

formation results shown in this thesis (and at the conference), and their independent verification by means of sequencing the VR1- and VR2-containing region of the packaged retroviral RNA, these conclusions were clearly inaccurate. The author attributed the discrepancy of results to the possible use of a different and less precise substitution strategy employed by the other group, but the investigators were not prepared to divulge experimental details of how they had substituted VR1. This was unfortunate, since a comparison of their construct with $pJS_{21}$/56A1VR1 would have revealed subtle differences, and those differences could have been implicated in the particle release defect.

In a subsequent review, Palmarini *et al.* (2004) mention unpublished studies in which they also substituted the enJS56A1-derived VR1 region (amino acids 89–142 of Gag) into the same infectious molecular JSRV clone used in this study, *viz.* $JSRV_{21}$. This chimera did not support particle release into the transfection supernatant. In contrast, the precise VR1 substitution (amino acids 112–132) in pJS21/56A1VR1 produced virus particles as shown. Alignment of the deduced amino acid sequences of the two chimeras (Fig. 3.19) indicates that the 89-142 chimera would differ from the 112–132 chimera by only two additional enJS56A1-derived amino acids at positions 98 (Cys/Arg) and 102 (Val/Leu). These are highlighted in Figure 3.19. This suggested that either Cys98 or Val102 (or both in combination) are involved in the particle release defect of enJS56A1, potentially along with VR1 sequences. It was noteworthy, however, that all other known enJSRV Gag sequences resemble the exogenous isolates in these positions, *i.e.* they have Arg98 and Leul02. If the Cys98 and/or Val102 were indeed involved in the enJS56A1 particle release defect, this implied that the defect is not a general one for enJSRVs. These conclusions were published along with the substitution strategy and the particle formation results (Hallwirth *et al.*, 2005). After this work was accepted for publication, Mura *et al.* (2004)

reported that the combined mutation of R98C and L102V indeed led to loss of particle production for wild-type JSRV, and that the other endogenous Gag proteins do not show the particle production defect of enJS56A1, thereby providing experimental evidence to support the above conclusions and strongly suggesting VR1's lack of specific involvement in the particle release defect.

#### 3.4.1.4 Investigation of the enJS56A1 dominant negative block of particle release

This study extended the findings reported at a conference, which showed that enJS56A1 inhibits the release of exogenous JSRV particles, by narrowing the region responsible down to the first two thirds of enJS56A1 *gag*. Furthermore, it was demonstrated that the addition of a three-fold excess of pCMV2JS$_{21}$ relative to a chimeric construct containing the first two thirds of enJS56A1 *gag* did not rescue the ability of the exogenous construct to release particles. The first two thirds of enJS56A1 *gag* also inhibited particle release by the chimeric constructs containing the enJS56A1-derived VR1 and/or VR2.

A possible explanation was put forward by the author (Hallwirth *et al.*, 2005): The enJS56A1 Gag protein might have a defective interaction with a component of the cell sorting machinery that is responsible for transport and budding of retroviral proteins (Bouamr *et al.*, 2003; von Schwedler *et al.*, 2003). If, therefore, enJS56A1 Gag protein were trapped in an intracellular compartment and unable to undergo normal transport to the cell surface, then it would likely inhibit wild-type exogenous JSRV particle release if they are both expressed in the same cell, through Gag–Gag protein interactions. These Gag–Gag interactions typically are in the nucleocapsid portion of Gag (Swanstrom and Wills, 1997),

which is highly conserved between endogenous and exogenous JSRVs. As a result, it was not surprising that enJS56A1 Gag also inhibited the release of the VR1 and VR2 chimeras. This hypothesis has very recently been experimentally addressed by another group of investigators and found to be accurate (Murcia *et al.*, 2007). They determined that the entire enJS56A1 Gag is required to prevent exogenous JSRV particle release and that JSRV Gag normally accumulates pericentrosomally. They observed co-localisation of enJS56A1 and exogenous JSRV Gag and conclude that the blocking effect exerted by enJS56A1 Gag on JSRV is based on Gag-Gag interactions that result in abnormal cellular localisation of the latter.

#### 3.4.1.5 VR1- and VR2-substituted chimeric particles are infectious

The primary goal of this aspect of the study was to create relevant molecular tools that could be used to elucidate the biological significance of VR1 and VR2. These tools constituted the VR1- and VR2-substituted chimeric constructs that have been described. In order to be used for *in vivo* studies, these chimeric constructs (or at least one of the three) was required to form intact retroviral particles. This turned out to be the case for all three of them. However, the final requirement for these particles was that they needed to be able to infect host cells, meaning they had to be able to interact with the relevant cell surface receptor(s) to enter the cells, reverse transcribe their genomic RNA and integrate that into the DNA of the host. This was assayed *in vitro* by infection of sheep choroid plexus cells, and the particles were found to be infectious, since their integrated DNA could be detected in the genomic DNA of the infected host cells several passages post-infection. Appropriate controls had been included in the design of the experiment to exclude the possibility that

the results were an artefact of plasmid carry-over. The chimeric particles are therefore suitable for *in vivo* infection studies, and since all three constructs gave rise to infectious particles, the effects and significance of both VR1 and VR2, as well as any possible combined effects of the two, can be tested.

### 3.4.2 Critical appraisal of JSRV Env *in vitro* transformation studies

The subject of JSRV transformation will be elaborated upon in detail, owing not only to its important contribution to cancer research, but also to the fact that our collaborators' pioneering research of JSRV transformation followed directly from our group's discovery of the JSRV variable region 3. For this reason, our group has always followed these developments very closely, the author having even been present at the CRI/UCI in 2000 when the first *in vitro* JSRV transformation assays were carried out. He was also there again in 2003, and party to all the work performed there that was subsequently published. We have also maintained close ties with other groups working on various aspects of JSRV transformation, to the degree where many results were made available to us at the pre-publication stage. Most importantly, detailed insight into the current understanding of JSRV transformation was required of the author to maintain a scope of his work in the context of the direction in which the field of JSRV research is moving.

The future continuation of the author's work described thus far is intricately linked with the developments pertaining to JSRV oncogenesis research *in vivo*. The study that comprises the author's work was designed not just as a terminal project for the sake of generating relevant and publishable data that can form the experimental core of a thesis. It

was designed in a broader context, to be continued (either by the author at postdoctoral level, or by other researchers) and to provide the tools, knowledge and insight required for this continuation. The broader project aims were to render it directly compatible with *in vivo* pathogenesis studies, once the tools for this had been developed. Since the pathogenesis of jaagsiekte manifests as a neoplasm, the author was required to keep abreast of the developments pertaining to JSRV transformation. This task thus constituted an integral theoretical (as opposed to laboratory-based) aspect of his research, on which he was required to deliver regular presentations to his peers, as developments in JSRV transformation research took place. In this chapter, the recent progress that has been made in elucidating the putative steps involved in JSRV transformation will be critically appraised in chronological order, covering the time span during which the author's practical work was ongoing. Not all JSRV Env transformation work involves VR3 directly, but those studies that do cannot be described in isolation while still providing a coherent overall context of the research. Some of the most recent developments pertaining to JSRV transformation will be discussed separately from the critical appraisal of the initial ground-breaking *in vitro* transformation work, since the author was no longer involved in this latest work in the way he had been during the initial studies.

#### 3.4.2.1 JSRV Env can transform immortalised rodent fibroblasts

As described in Chapter 1, conclusive evidence of JSRV being the sole agent of transformation in OPA was first published by the CRI/UCI, in collaboration with two other international laboratories (Palmarini *et al.*, 1999a). However, this study could not yet address by what possible mechanism(s) JSRV might lead to cellular transformation. In

2000, the CRI/UCI was the first group to embark on *in vitro* transformation studies using JSRV expression constructs, and their landmark results were published the next year (Maeda *et al.*, 2001). In this study, Maeda *et al.* transfected mouse NIH 3T3 cells with plasmid constructs $pJSRV_{21}$ and $pCMV2JS_{21}$ (Palmarini *et al.*, 1999a). The pcDNA3.1(-) vector was used as a negative control because it contains the same CMV immediate early promoter as $pCMV2JS_{21}$, but lacks JSRV sequences. Transfected cells were maintained in culture for four to five weeks. Two weeks after transfection with $pCMV2JS_{21}$, foci of transformed cells became visible. The transformed cells were recognised by their fusiform shape and loss of contact inhibition, causing cells to pile on top of each other. Foci were counted 28 days after transfection, and it was noted that the transformation efficiency of $pJSRV_{21}$ was much lower than that of $pCMV2JS_{21}$, owing to the fact that the JSRV LTR is a poor promoter / enhancer in NIH 3T3 cells. Transformed cells were also shown to exhibit anchorage independence. Furthermore, these results were confirmed by transforming Rat6 cells in culture, though at much lower efficiency than NIH 3T3 cells.

Maeda *et al.* (2001) established transformed cells lines, from which they were able to isolate JSRV plasmid DNA, as well as JSRV RNA. Together, the above results indicated that some part(s) of the JSRV genome is (are) able to transform NIH 3T3 cells in culture. To address which gene(s) might be involved, two stop codons were introduced into the *orf-x* gene without affecting the overlapping *pol* amino acid sequence. The resulting construct, $pCMV2JS_{21}\Delta$orf-x, transformed NIH 3T3 cells with the same efficiency as $pCMV2JS_{21}$, from which the investigators concluded that the *orf-x* gene product is not directly involved in cell transformation. They proceeded to make expression constructs for each of the coding genes of JSRV and tested them for their ability to transform NIH 3T3 cells (H. Fan, personal communication). Only the Env-expressing construct transformed cells. In Maeda

*et al.* (2001), this construct is called pCMV3JS$_{21}$ΔGP, because it is lacking *gag* and *pol*, and it is shown to induce cellular transformation at efficiency comparable to a plasmid expressing the activated c-*ras* proto-oncogene from human T24 bladder carcinoma cells driven by a murine leukaemia virus LTR.

The investigators further point out that the transformation efficiency of pCMV3JS$_{21}$ΔGP (*i.e.* Env expressed by itself) is higher than that of the full-length clone pCMV2JS$_{21}$. This is true when looking at their table of results, in that the former induced an average of 20 and the latter an average of 16.3 foci under the same experimental conditions. However, what the investigators appear to have disregarded is the fact that they used the same amount of DNA (28 μg) in every transfection, but that the ΔGP plasmid is only approximately half the size of the full-length construct. This means that the transfection dose of 28 μg of the ΔGP construct contains double the number of plasmid copies, and would thus be expected to produce about twice the number of foci, which is certainly not the case. One could consider this to indicate that some genetic elements missing from the ΔGP construct, but contained in the full-length pCMV2JS$_{21}$ construct, significantly enhance the transforming activity of the JSRV *env* gene product.

Using northern blotting, Maeda *et al.* (2001) found that two forms of JSRV *env* are expressed in transfected (as well as transformed) cells, *viz.* a full-length 2.5 kb transcript, as well as a prematurely polyadenylated, and therefore truncated, 1.2 kb transcript referred to as *tr-env*. When expressed by itself, *tr-env* (which essentially encodes only the SU protein and is missing TM, including VR3) was incapable of transforming NIH 3T3 cells. Since rodent cells lack a functional JSRV receptor (Rai *et al.*, 2000), the observed transformation associated with full-length Env could not have been as a result of cell-to-

cell spread of infectious virus, so the investigators concluded that JSRV Env, either by itself or in combination with the *tr-env* product, is able to transform NIH 3T3 cells. This constituted the only other report in addition to a group working with vascular endothelial tumours and avian haemangioma virus (Alian *et al.*, 2000), that a normally functional retroviral structural protein could have direct oncogenic potential.

### 3.4.2.2 Hyal2 is the cellular receptor for JSRV

Appearing at the same time as the study of Maeda *et al.* (2001) was a report that identified the product of a candidate lung and breast cancer tumour suppressor gene, human Hyal2, as the cellular receptor of JSRV in cell culture (Rai *et al.*, 2001). Other animals, including sheep, have closely related orthologues of human Hyal2. It was shown that Hyal2 is a glycosylphosphatidyl-inositol (GPI)-anchored cell-surface protein. Since some other GPI-anchored proteins mediate mitogenic responses, the investigators speculated that an interaction of JSRV Env with Hyal2 might be involved in JSRV transformation. They also used an existing JSRV Env expression construct, called pSX2.Jenv (Rai *et al.*, 2000), to successfully transform 208F rat embryo fibroblasts *in vitro*, thereby confirming the results of Maeda *et al.* (2001). This construct is driven by a murine leukaemia virus (MLV) LTR, with the *env* gene being derived from the infectious molecular clone $JSRV_{JS7}$ (DeMartini *et al.*, 2001). Even though Rai *et al.* (2001) showed that the rodent orthologues of Hyal2 do not efficiently mediate JSRV entry, they point out that this does not preclude the possibility of some other productive interaction between the Hyal2 orthologue in rodent fibroblasts used in the transformation assays, and the JSRV Env protein.

Taken together, the results of Maeda *et al.* (2001) and Rai *et al.* (2001) prompted the suggestion of two possible models of transformation involving Hyal2 and JSRV Env (Maeda *et al.*, 2001; Rosenberg, 2001). The first was that the binding of Env to the putative tumour suppressor Hyal2 would interfere with its normal negative growth regulatory activity. Secondly, binding of Env to Hyal2 could result in positive growth stimuli. Another possibility was that JSRV Env interacts with another intracellular or surface cell protein to induce transformation.

### 3.4.2.3 A PI3K binding motif in the cytoplasmic tail of JSRV Env seems essential for transformation

The finding of Maeda *et al.* (2001) that JSRV Env can transform NIH 3T3 cells were elaborated upon by the same group of investigators via the construction of various envelope chimeras, using sequences derived from $JSRV_{21}$ and enJSRV loci enJS56A1 and enJS5F16 (Palmarini *et al.*, 2001b). Their construct pSUxTMen (Fig. 3.20), consisting of

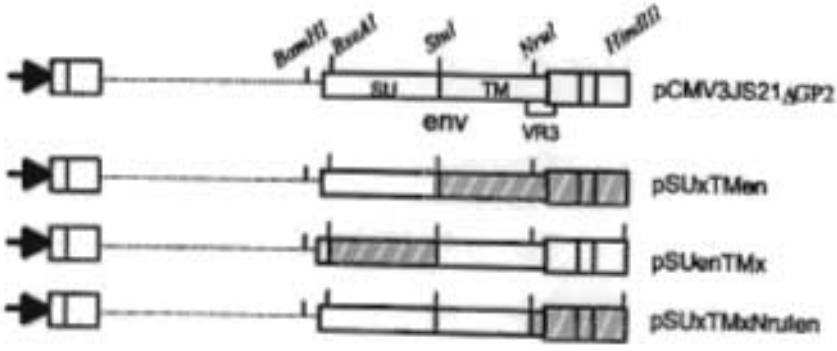



**Figure 3.20 Schematic representation of chimeric constructs using $JSRV_{21}$ and endogenous JSRV sequences.** Adapted from Palmarini *et al.* (2001b).

the exogenous surface and endogenous transmembrane domain, was unable to transform NIH 3T3 cells, indicating that parts of TM are needed for transformation. Progressively smaller substitutions indicated a need for VR3, and within VR3, for the carboxy-terminal 36 amino acids downstream of an *Nru*I site in VR3, the corresponding construct being

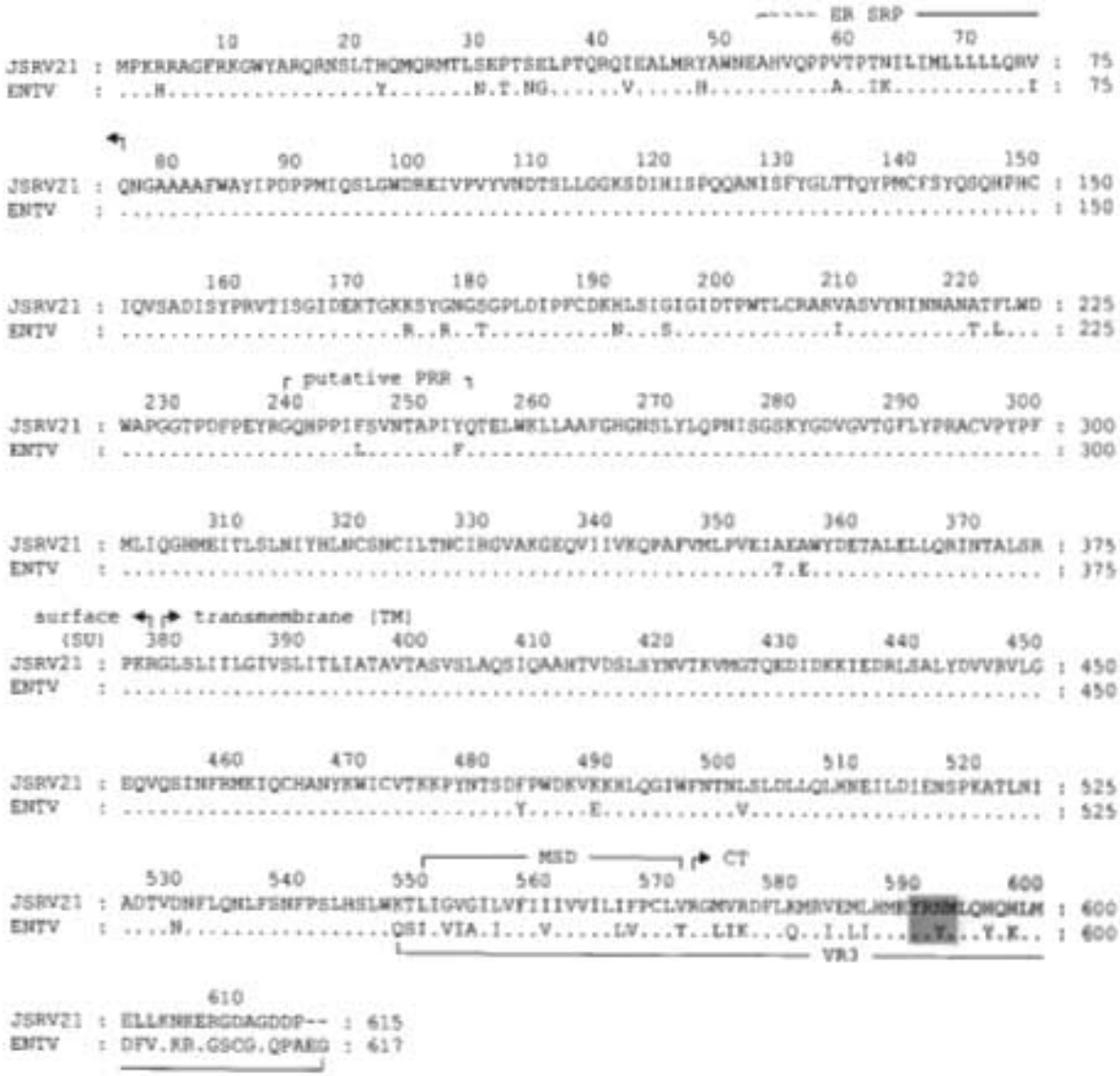



**Figure 3.21 Amino acid alignment of the $JSRV_{21}$ and ENTV envelope protein sequences.** Annotations (Chow *et al.*, 2003; Dirks *et al.*, 2002; Hofacre & Fan, 2004; York *et al.*, 1992) indicate the positions of regions within Env. Env is primarily divided into the surface (SU) and transmembrane (TM) proteins. The 5' endpoint of the endoplasmic reticulum signal recognition peptide (ER SRP) has not been assigned with certainty. PRR, proline rich region; MSD, membrane spanning domain; CT, cytoplasmic tail; VR3, variable region 3. A putative PI3K binding domain, YXXM, is highlighted.

named pSUxTMxNrulen (Fig. 3.20). It was noted that the reciprocal chimera pSUenTMx also did not transform NIH 3T3 cells, possibly indicating a further transformation requirement within SU. The other postulated explanation for this observation was that all the chimeras containing endogenous SU sequences are expressed at far lower levels than those containing exogenous SU sequences. This work was referred to in Palmarini *et al.* (2001b), and separately published later (Palmarini *et al.*, 2002).

Having narrowed the critical determinants of transformation in NIH 3T3 cells down to the carboxy-terminal 36 amino acids of the exogenous VR3 (essentially the membrane-spanning domain and cytoplasmic tail of the transmembrane protein; Fig. 3.21), Palmarini *et al.* (2001b) compared all known JSRV sequences (both endogenous and exogenous) corresponding to this region. They found that all exogenous JSRVs (including ENTV) contain a YXXM motif, starting at position 590 in $JSRV_{21}$, that is missing from enJSRVs (Fig. 3.22). Also, most exogenous JSRV isolates have a YXN motif, starting at the same

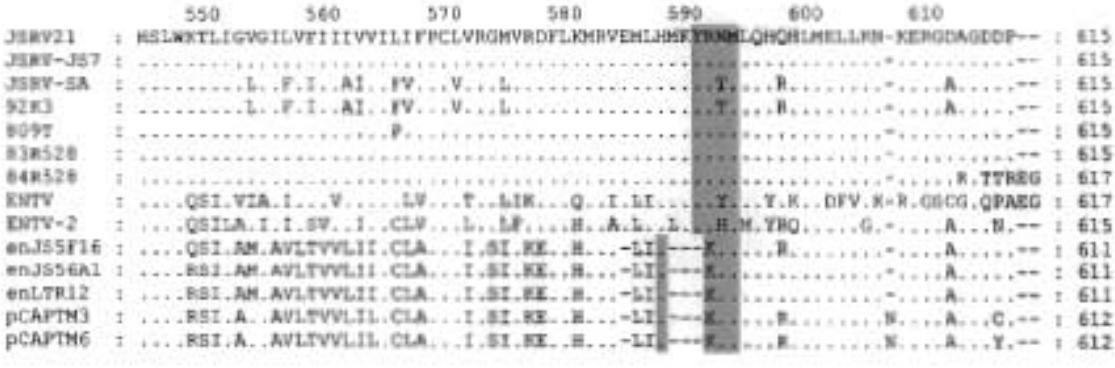



**Figure 3.22 Amino acid alignment of JSRV VR3, highlighting the $Y^{590}$-X-X-$M^{593}$ motif in $JSRV_{21}$, as well as its corresponding residues in other JSRV and JSRV-related sequences.**

position. The investigators do not mention that JSRV sequences of African origin (JSRV-SA and 92K3) and ENTV lack this motif (Fig. 3.22). YXXM is a potential binding site for the regulatory alpha subunit (p85) of phosphatidylinositol 3-kinase (PI3K), while YXN motifs bind the growth factor receptor binding protein 2 (Grb-2). Since both these proteins

can be involved in cellular transformation processes, Palmarini *et al.* (2001b) mutated both the motifs to determine if such mutations would abolish transformation. Mutation of $Y^{590}$ did (whereas mutation of other tyrosine residues in VR3 either had no effect, or just reduced transformation efficiency), as did mutation of M593. Mutation of N592 did not; in fact, when this residue was mutated to threonine, transformation was markedly enhanced. (Once again, the investigators did not point out that exogenous JSRV sequences of African origin naturally have threonine in position 592 – see Figure 3.22.)

Palmarini *et al.* (2001b) concluded that PI3K binding to YXXM is essential for transformation of NIH 3T3 cells, whereas Grb-2 binding and subsequent signalling through the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, is not. They did not, however, exclude the possibility of the MAPK pathway being activated differently as part of JSRV transformation. To further support the involvement of PI3K in JSRV transformation, Palmarini *et al.* (2001b) showed that transformed NIH 3T3 cells contain phosphorylated Akt, a protein kinase that is activated via another intermediate by PI3K, whereas parental NIH 3T3 cells contained only the non-activated / non-phosphorylated form of Akt. They also call attention to the fact that the activation of the PI3K/Akt pathway might only be one of several steps required for transformation *in vivo*.

#### 3.4.2.4 ENTV Env has the same transforming potential as JSRV Env

Not surprisingly, ENTV Env was shown to transform NIH 3T3 cells as well (Dirks *et al.*, 2002); in fact, it does so at the same efficiency as JSRV Env. Dirks *et al.* (2002) further report that JSRV and ENTV both use ovine Hyal2 as receptor, and argue against a

receptor-mediated transformation mechanism in JSRV and ENTV on the basis that Hyal2 is ubiquitously expressed in many tissues, and that JSRV even integrates into several different cell types (Holland *et al.*, 1999; Palmarini *et al.*, 1996b; Palmarini *et al.*, 1999b). Yet, it is only oncogenic in the epithelial cells of the lower airways. Dirks *et al.* (2002) speculate that the differences in U3 between JSRV and ENTV lead to different tissue-specific expression patterns, and that JSRV and ENTV Env-mediated transformation is dependent upon active expression, rather than the binding of Env to the JSRV / ENTV receptor.

The results of Dirks *et al.* (2002) and Palmarini *et al.* (2001b) were extended by a report that ENTV Env can transform rat 208F cells (in addition to mouse NIH 3T3 cells); that the tyrosine residue in the YXXM motif of ENTV Env (equivalent to JSRV Env $Y^{590}$) is equally indispensable to transformation; and that ENTV Env-transformed rodent (NIH 3T3 and rat 208F) cells are characterised by phosphorylated Akt (Alberti *et al.*, 2002). The latter observation addressed the concern that Akt activation might be specific either to JSRV transformation, or to transformation of NIH 3T3 cells only.

Whereas previous studies had not determined if Akt phosphorylation was indeed PI3K-dependent, Alberti *et al.* (2002) demonstrate a dose-dependent inhibition of Akt phosphorylation by a PI3K-specific inhibitor (LY294002). The investigators also established NIH 3T3 cell lines constitutively expressing either JSRV Env, or JSRV Env with $Y^{590}$ mutated, and found the former to be characterised by phosphorylated Akt whereas the latter contained only unphosphorylated Akt, thereby further linking JSRV Env $Y^{590}$ to rodent cell transformation and PI3K/Akt phosphorylation *in vitro*. Furthermore, they detected phosphorylated Akt in the JS-8 cell line, which is derived from the tumour of

a sheep with naturally occurring OPA; even this phosphorylation could be inhibited by LY294002. One matter that the investigators did not address (and it becomes a point of note at a later stage) is that rat 208F cells transfected with an ENTV Env $Y^{590}$ mutant exhibit a very limited degree of transformation – approximately 1% compared with wild-type ENTV Env. Although 1% efficiency of transformation might be considered negligible in certain contexts, it certainly is not when viewed in light of the suggestion that JSRV might be suitable as a gene transfer vector for differentiated epithelial cells of the lung and that the mutation of Env $Y^{590}$ would uncouple its unwanted transforming potential from its ability to mediate viral entry (Palmarini *et al.*, 2001b). Even a 99% reduction of transforming potential would still render Env $Y^{590}$-mutated JSRV an unsuitable, high-risk gene transfer vector.

#### 3.4.2.5 JSRV Env can transform immortalised chicken fibroblasts

JSRV Env can also transform the DF-1 immortalised chicken fibroblast cell line (Allen *et al.*, 2002). Here, the investigators used avian sarcoma-leukaemia virus (ASLV)-derived vectors to express different genomic components of $JSRV_{JS7}$ upon transfection of DF-1 cells. They found that neither the o*rf-x* gene product nor SU by itself could transform DF-1 cells, whereas Env did. Supernatants of cells transfected with the JSRV Env-expressing vector were also used to infect DF-1 cells, with the same results. Furthermore, transfected cells were inoculated subcutaneously into nude mice, which led to tumour formation.

Deletion of the 33 carboxy-terminal amino acids of Env abolished transformation of DF-1 cells (Allen *et al.*, 2002), which indicated that this region either contains elements required

for transformation, or that it is merely needed for the functionally correct conformation of Env. Expression of this truncated form of Env was not inhibited relative to full-length Env, eliminating this as a possible explanation. The carboxy-terminal 33 amino acids of $JSRV_{JS7}$ contain the YXXM motif that was shown to be essential for transformation of rodent fibroblasts by both JSRV and ENTV Env (Alberti *et al.*, 2002; Palmarini *et al.*, 2001b). Allen *et al.* (2002) mutated both the tyrosine and methionine residues in their Env expression construct and found, surprisingly, that neither of these mutations significantly reduced the transformation efficiency compared to the wild-type Env. The investigators discuss this apparent contradiction with the results of the previously published reports by suggesting that PI3K might be activated in both rodent and avian cells transformed by JSRV Env, but that PI3K does not interact directly with the Env protein. It is worth noting, even if the possible significance of these comparisons cannot be evaluated here, that Allen *et al.* (2002) used $JSRV_{JS7}$ in avian DF-1 cells, and that they mutated the tyrosine residue of YXXM to either alanine or serine and the methionine to either valine or leucine. Palmarini *et al.* (2001b) and Alberti *et al.* (2002) collectively used $JSRV_{21}$ and ENTV in rodent fibroblasts, and they mutated the tyrosine of YXXM to aspartic acid or phenylalanine and the methionine to threonine. The use of $JSRV_{21}$ *vs.* $JSRV_{JS7}$ *per se* is not likely to explain any differences between studies relating to JSRV Env transformation, as the amino acids sequences of these two isolates are identical.

#### 3.4.2.6 Role of Hyal2 in JSRV Env transformation

If Hyal2 is indeed a tumour suppressor gene, then the binding of JSRV Env to Hyal2 might inhibit Hyal2 and thus lead to oncogenic transformation (Liu *et al.*, 2003a). In support of

their hypothesis, Liu *et al.* (2003a) began by demonstrating physical binding of JSRV SU to Hyal2 orthologues and that Hyal2 does mediate JSRV entry into cells. Previously, this had only been inferred from genetic studies. They developed NIH 3T3 and 208F cell lines that express human Hyal2, and showed that these cell lines are considerably less sensitive to JSRV Env transformation than parental NIH 3T3 and 208F cells that do not over express human Hyal2. When Liu *et al.* (2003a) transduced JSRV Env-transformed NIH3T3 cell lines with vectors expressing human Hyal2, 80% of the cells reverted to their normal non-transformed phenotype.

Liu *et al.* (2003a) added a FLAG tag (DYKDDDDK) to the 3' end of the Env coding region in their JSRV Env expression construct. Using an anti-FLAG antibody, this allowed them to monitor the levels of Env protein in transfected NIH 3T3 cells. They found a significant reduction in JSRV Env levels in those cells expressing human or rat Hyal2, more so for human than for rat Hyal2. Mouse Hyal2 did not reduce levels of JSRV Env, even when over expressed. Similarly, the above results, *viz.* binding of Hyal2 to SU, suppression of Env transformation by Hyal2 and the reversal of transformed phenotype all pertained most strongly to human Hyal2, followed by rat Hyal2, and did not apply to mouse Hyal2. This means that JSRV Env transforms mouse NIH 3T3 fibroblasts without any discernible interaction with Hyal2 in these cells, making it very unlikely that mouse Hyal2 is involved in JSRV Env transformation of NIH 3T3 cells (Liu *et al.*, 2003a). The reduction of JSRV Env levels in cells expressing human or rat Hyal2 does not support the notion of a specific tumour suppressor activity of Env transformation by Hyal2. Rather, it would seem that a more generalised effect of Hyal2-mediated degradation of Env is responsible for the reduction of transformation in those cells expressing human or rat Hyal2. In the discussion of their results, the investigators did not mention the possibility of

interaction between Hyal2 and the extracellular domain of JSRV TM. In view of all the findings to this point, such an interaction could still allow for a role of Hyal2 in JSRV Env-mediated transformation, even of NIH 3T3 cells.

All JSRV Env *in vitro* transformation studies thus far had used immortalised fibroblast cell lines, owing to their ease of transfection and the ability to employ established transformation assay protocols. However, the target cells of JSRV transformation *in vivo* are epithelial cells, so Danilkovitch-Miagkova *et al.* (2003) investigated JSRV Env transformation *in vitro* using an immortalised non-transformed human bronchial epithelial cell line, BEAS-2B. They found that transfection with a plasmid encoding an influenza haemagglutinin (HA) tag and JSRV Env led to stable expression of JSRV Env in these cells and to formation of transformed foci after six weeks.

In BEAS-2B control cells, Hyal2 is associated with an inactive / dormant form of the receptor tyrosine kinase RON, as shown by coimmunoprecipitation and western blotting (Danilkovitch-Miagkova *et al.*, 2003). In BEAS-2B cells that had been transformed by JSRV Env, however, RON was no longer associated with Hyal2, and it was activated. The investigators speculate that this activation of RON occurs by means of autophosphorylation. They show that the activation of RON in Env-transformed cells is associated with constitutive activation of Akt and mitogen-activated protein kinase (MAPK), so that activation of the Akt and MAPK pathways would drive proliferation and transformation, and maintain the transformed phenotype of BEAS-2B cells. The investigators point out that both Akt and MAPK had previously been shown to be activated directly by kinase-active RON. The ectopic over expression of "kinase-dead" RON (kdRON), however, could reverse Env-mediated transformation (Danilkovitch-Miagkova

*et al.*, 2003), presumably because it abrogates activation of the Akt and MAPK pathways. Over expression of Hyal2 was also able to reverse transformation in this system. Taken together, the above results meant that RON activation is essential for transformation and its maintenance in BEAS-2B cells, and the investigators concluded that Hyal2 negatively regulates the function of RON receptor tyrosine kinase in bronchial epithelial cells.

BEAS-2B cells are difficult to transfect, so Danilkovitch-Miagkova *et al.* (2003) continued their studies in human embryonic kidney and canine kidney cells. In these cell lines, they confirmed that JSRV Env induced ligand-independent RON and MAPK activation and that both are reduced by co-expression of Hyal2. In both cell lines, Env was NOT tyrosine-phosphorylated. Such tyrosine phosphorylation would be required by the model of rodent fibroblasts transformation proposed by Palmarini *et al.* (2001b) and Alberti *et al.* (2002), involving Env activation of the PI3K/Akt pathway. Danilkovitch-Miagkova *et al.* (2003) observed that RON tyrosine kinase phosphorylation is directly dependent on Env

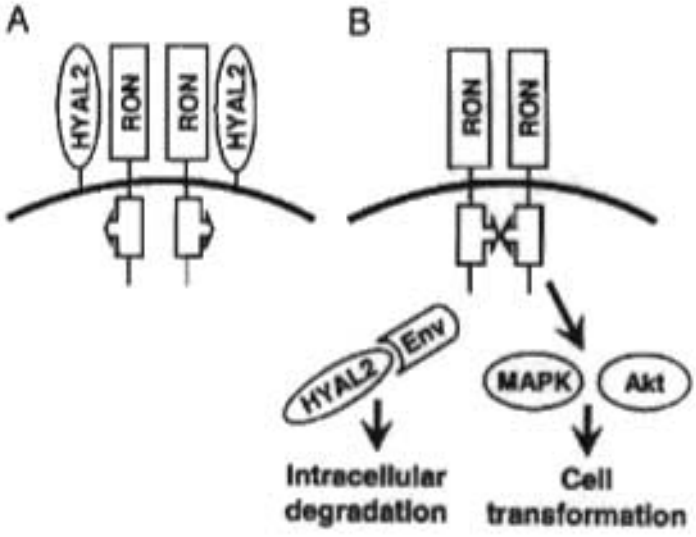



**Figure 3.23 Model of JSRV Env-mediated transformation of RON-expressing cells.** A) Hyal2 negatively regulates the activity of RON kinase dimmers. B) JSRV Env competitively binds Hyal2, removing it from its association with RON. RON autophosphorylates and activates MAPK and Akt, leading to cellular transformation. (Danilkovitch-Miagkova *et al.*, 2003)

expression and that Env competes with RON for binding Hyal2. Also, the complex formed by Hyal2 and Env is degraded intracellularly. The scenario they propose for epithelial cells is that RON is expressed on the cell surface as an inactive dimer, the activation of which is prevented by its association with Hyal2 (Fig 3.23A). Competitive binding of Hyal2 by JSRV Env mediates viral entry, and subsequently expressed Env sequesters Hyal2. The Env/Hyal2 complex is degraded via a cellular proteasomal mechanism. The removal of Hyal2 from its association with RON renders the latter active, which induces activation of oncogenic pathways involving Akt and MAPK (Fig 3.23B). The activation of both these kinases was shown to be Env-dependent in this study.

In further support of their proposed mechanism, Danilkovitch-Miagkova *et al.* (2003) showed that RON is activated in some human BAC cell lines and in one lung adenocarcinoma. They also point out that the over expression of RON in the distal lung epithelial cells of transgenic mice can induce peripheral adenocarcinomas (Chen *et al.*, 2002), thereby providing more substantiation for a role of RON in lung cancer. They agree that their model cannot apply to NIH 3T3 cells transformed by JSRV Env, because mouse Hyal2 does not interact with JSRV Env, and NIH 3T3 cells do not even express Stk (the mouse orthologue of RON).

#### 3.4.2.7 JSRV Env transformation of rodent fibroblasts does not require the SU domain

In rat 208F cells, the involvement of Hyal2 was all but excluded by the finding that these cells could be transformed by JSRV Env in the absence of its putative receptor binding

domain (RBD) (Chow *et al.*, 2003). The investigators had first created viral particles based on MMuLV, pseudotyped with JSRV/MMuLV or JSRV/MMTV chimeric Env genes. Some of these were able to induce morphological changes in 208F cells, but the only chimeric Env gene that supported transformation contained the leader peptide, RBD and proline-rich region from MMuLV, while retaining the remainder of SU and the whole of TM from JSRV (Fig 3.24A). Hyal2 was unable to mediate viral entry of these particles, indicating that the receptor binding domain pertaining to Hyal2 had been successfully replaced. Chow *et al.* (2003) then showed that a JSRV Env expression construct from which the putative RBD had been deleted (JSEΔrbd; Fig. 3.24B) pseudotyped particles that were incapable of infecting cells expressing Hyal2, indicating that they cannot interact with Hyal2. The same construct, when used to transfect 208F cells, was able to transform them at about one third the efficiency of wild-type JSRV Env, demonstrating that Hyal2 is not required for JSRV Env transformation of rodent fibroblasts.

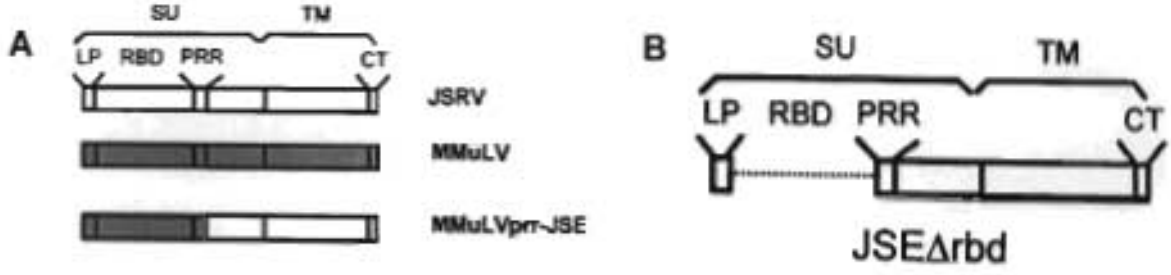



**Figure 3.24 Env expression constructs used by Chow *et al.* (2003) to determine lack of involvement of Hyal2 in transformation of rodent fibroblasts** (adapted from Chow *et al.*, 2003). A) Parent sequences for derivation of chimeric construct capable of 208F cell transformation, and structure of resulting construct. B) JSRV Env expression construct lacking the receptor binding domain.

From the results of the MMuLV/JSRV Env chimeras and the deletion of the JSRV RBD, the elements responsible for JSRV transformation could be localised to the carboxy-terminal third of SU, or TM. Chow *et al.* (2003) narrowed this down further by creating

progressive amino-terminal deletion constructs of TM, linked to a myristoylation signal (Myr) at the amino terminus so that the gene products would be localised to the cell membrane. The JSRV Env cytoplasmic tail (without which transformation does not occur) by itself was not able to transform 208F cells (Fig. 3.25, pMyr-JSE7061), nor were several progressively larger TM constructs. The smallest construct that did support transformation expressed the carboxy-terminal 141 amino acids of JSRV Env (Fig. 3.25, pMyr-JSE6770). The transformation efficiency was one to two thirds that of wild-type JSRV Env, and transformed cells were shown to contain activated Akt. Furthermore, when the myristoylation signal was omitted (Fig. 3.25, pΔMyr-JSE6770), transformation was abrogated, indicating a need for membrane localisation. The transmembrane domain of JSRV Env is therefore a major determinant of transformation, and 208F cells were transformed in the absence of JSRV SU, provided the TM protein was localised to the cell membrane.

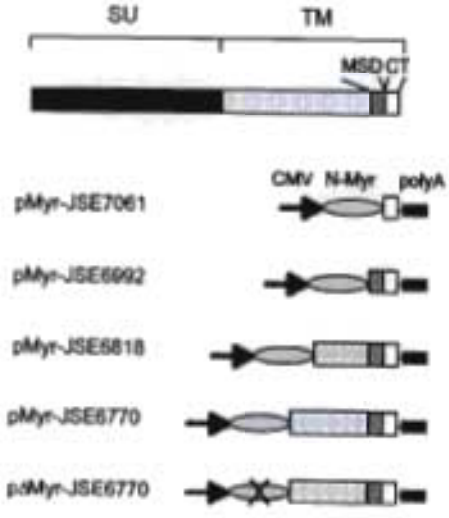



**Figure 3.25 Myristoylated JSRV TM expression constructs used by Chow *et al.* (2003) to narrow down the JSRV Env transformation requirements to the carboxy-terminal 141 amino acids of TM.**

### 3.4.2.8 PI3K binding domain in CT of JSRV Env is not required for transformation of rodent fibroblasts

The replacement of the JSRV Env cytoplasmic tail (CT) with the cytoplasmic domain of either MLV or HIV-1 abrogates transformation of 208F cells (Liu *et al.*, 2003b), confirming earlier findings that CT is indispensable to JSRV Env transformation. In contrast with the finding by Palmarini *et al.* (2001b) that the tyrosine and methionine residues in the YXXM motif of JSRV Env are essential for transformation of NIH 3T3 cells, Liu *et al.* (2003b) reported that both these residues can be mutated in JSRV and ENTV and still transform 208F as well as NIH 3T3 cells, albeit at reduced efficiency and sometimes only after transfected cells were left to grow for longer periods of time. As mentioned earlier in this chapter, Alberti *et al.* (2002) had already shown low levels of 208F transformation by an ENTV Env $Y^{590}$ mutant in one of their tables of results, but had failed to take this into account in the discussion of their results. These findings by Liu *et al.* (2003b) correlate with those of Allen *et al.* (2002), who had shown that, in the transformation of the chicken fibroblast cell line DF-1, both the tyrosine and methionine residues of YXXM are entirely dispensable.

Liu *et al.* (2003b) confirmed that Akt is phosphorylated in JSRV- and ENTV-transformed 208F cells, and showed that it is also kinase-active. They found that this activity was completely inhibited by the PI3K-specific inhibitor LY294002, thereby confirming the results of Alberti *et al.* (2002) that had shown Akt activation in transformed cells to be PI3K dependent. However, in contrast to Alberti *et al.* (2002) who had linked this activation of the PI3K/Akt pathway directly to $Y^{590}$, Liu *et al.* (2003b) showed that cells transformed (at lower efficiency) by the various constructs mutated in the YXXM motif

are still characterised by Akt phosphorylation and Akt activity, which was inhibited by LY294002. LY294002 could also reverse the transformed phenotype of Env-transformed rodent fibroblasts, regardless of whether the constructs used for transformation contained the YXXM motif or not.

Liu *et al.* (2003b) were unable to detect phosphorylated tyrosine in the Env proteins expressed by JSRV- and ENTV-transformed 208F cell lines. Such tyrosine phosphorylation would be required at YXXM if it were to bind and activate PI3K. They also did not detect any binding association between JSRV Env and p85, the regulatory subunit of PI3K. Taken together, this means that Akt activation in transformed rodent fibroblasts is PI3K dependent, but that the PI3K/Akt pathway is activated indirectly, *i.e.* not via activation of PI3K by JSRV or ENTV Env.

In contrast with their JSRV transformation studies on epithelial cells, where they showed MAPK to be activated (Danilkovitch-Miagkova *et al.*, 2003), Liu *et al.* (2003b) did not detect activated / phosphorylated MAPK in transformed rodent fibroblasts. They conclude that MAPK is probably not involved in the transformation of rodent fibroblasts, despite the fact that the MAPK pathway can play a role in tumourigenesis, either by itself or in combination with the PI3K/Akt pathway. They also found no association of JSRV Env with Grb2, despite the presence of the potential binding motif YXN. As pointed out earlier in this chapter, however, the involvement of Grb2 seemed somewhat unlikely in any event, since the YXN motif is found in only some exogenous pathogenic JSRV isolates.

### 3.4.2.9 Primary chicken fibroblasts can be transformed by JSRV Env

So far, the cytoplasmic tail of JSRV Env had been shown to be essential for JSRV transformation of both NIH 3T3 and 208F cell lines (Chow *et al.*, 2003; Liu *et al.*, 2003b; Palmarini *et al.*, 2001b). Despite several other differences pertaining to rodent *vs.* chicken fibroblasts, this absolute requirement of CT was shown to hold true in DF-1 cells, using a replication-competent avian retroviral vector expressing wild-type and mutated forms of $JSRV_{21}$ Env (Zavala *et al.*, 2003). This study also confirmed the results of Allen *et al.* (2002), which had demonstrated that the YXXM motif in the cytoplasmic tail of JSRV Env is dispensable to transformation of DF-1 cells, although Zavala *et al.* (2003) mutated the tyrosine to different residues compared with Allen *et al.* (2002). Zavala *et al.* (2003) extended previous findings by reporting that JSRV Env-transformed DF-1 cells contain phosphorylated Akt; this property is therefore shared by JSRV-transformed rodent, DF-1 and BEAS-2B cells (Danilkovitch-Miagkova *et al.*, 2003), as well as JS-8 cells (Alberti *et al.*, 2002). However, Zavala *et al.* (2003) did not find phosphorylated Akt in those DF-1 cells transformed by $Y^{590}$ mutants, which contrasts with the results of Liu *et al.* (2003b) using 208F cells.

All *in vitro* JSRV transformation assays to this point had used immortalised (yet non-transformed) cell lines, which had undergone genetic changes that would render them more susceptible to transformation compared to primary cells. Zavala *et al.* (2003) were the first to demonstrate JSRV transformation of primary cells, using primary chicken embryo fibroblasts (CEFs). In contrast with DF-1 cells, they found that Env $Y^{590}$ mutations reduce the degree to which CEFs are transformed (in terms of phenotypic changes), and the degree of reduction varies depending on which amino acid residue the tyrosine is mutated

to. Furthermore, Akt phosphorylation was only found in fully transformed CEFs, *i.e.* those that had been transformed by non-mutated JSRV Env. This indicates a role in efficient transformation, involving Akt activation, for $Y^{590}$.

Like Liu *et al.* (2003b), Zavala *et al.* (2003) showed that phosphorylated Akt in JSRV-transformed 208F cells is indeed kinase-active. They also extended their earlier results of dose-dependent inhibition of JSRV Env transformation by the PI3K-specific inhibitor LY294002 in NIH 3T3 cells (Alberti *et al.*, 2002) to include 208F cells. However, using various concentrations of the inhibitor, Zavala *et al.* (2003) found evidence of possible generalised toxicity of LY294002 in their experiments, and could therefore not rule out that the observed inhibition of transformation by LY294002 relates to this, rather than to the inhibition of the PI3K/Akt pathway. Finally, they tested ten OPA tumours from sheep and ten ENT (five from sheep and five from goats) tumours for Akt phosphorylation. All OPA tumours were negative, as were four of the ENT tumours. From all their results, Zavala *et al.* (2003) conclude that activation of the PI3K/Akt pathway plays an important role in JSRV transformation, but that it is not likely to be the pivotal event in this process.

#### 3.4.2.10 Evidence that indicates JSRV transformation of rodent fibroblasts does not involve PI3K

Maeda *et al.* (2003) used four different experimental approaches to show that JSRV transformation of rodent fibroblasts does not involve PI3K. The first two made use of dominant negative forms of the PI3K regulatory subunit (Δp85), which would still be able to bind phosphorylated YXXM motifs, but not be able to form dimers with the catalytic

subunit of PI3K. Transformation efficiency of NIH 3T3 cells was unchanged, and the transformed cells still contained phosphorylated Akt, indicating that Akt had been activated independently of PI3K (Maeda *et al.*, 2003). The next experiment involved the use fibroblasts derived from mice in which the genes encoding the p85α and p85β isoforms of the regulatory subunit of PI3K had been knocked out. These cells were also efficiently transformed by JSRV Env (Maeda *et al.*, 2003).

Finally, Maeda *et al.* (2003) found that, even though the PI3K-specific inhibitor LY294002 was able to inhibit JSRV Env transformation in NIH 3T3 cells, it inhibited transformation by v-*mos* (a PI3K-independent oncogene) to the same degree, indicating that the inhibition of transformation by LY294002 is likely to be a non-specific effect. Furthermore, LY294002 was unable to reverse the transformed phenotype of NIH 3T3 or Rat 6 cells transformed by JSRV Env (Maeda *et al.*, 2003). Both these observations stand in direct conflict with those of Liu *et al.* (2003b) who had shown that JSRV transformation of 208F cells could be reversed by LY294002, and that Fos-induced transformation of 208F cells (also PI3K-independent, like v-*mos*) was only very weakly reduced by LY294002. Liu *et al.* (2003b) had attributed this weak reduction to toxic effects of LY294002. A further contrast with the findings of Liu *et al.* (2003b) is that Maeda *et al.* (2003) still report the YXXM motif in the cytoplasmic tail of JSRV Env to be necessary for transformation of NIH 3T3 cells.

It might be worth noting at this point that there is a potentially important difference between the constructs used in the work by Liu *et al.* (2003b) and JSRV Env expression constructs from Hung Fan's and Massimo Palmarini's laboratories. Whereas the latter are driven by a human cytomegalovirus promoter / enhancer, the former are driven by a

murine leukaemia virus LTR, which is likely to be more efficient in mouse and other rodent cells. None of the publications pointed out this difference, even when commenting on and discussing the contrasting results that the different groups were reporting.

Owing to their continued finding that YXXM is necessary for transformation of rodent fibroblasts, Maeda *et al.* (2003) restored this motif in pCMV3ΔGP(NruIend), a construct that expresses JSRV Env, but with an endogenously derived cytoplasmic tail downstream of the *Nru*I site. Prior to restoration of YXXM, this construct did not transform NIH 3T3 cells. This was expected, as it is essentially the same as pSUxTMxNruIen described by Palmarini *et al.* (2001b) (Fig. 3.20). By replacing $His^{586}$ with Tyr, Maeda *et al.* (2003) restored the YXXM motif in this construct, but this did not rescue its ability to transform NIH 3T3 cells, meaning that residues other than the PI3K docking motif in the cytoplasmic tail of JSRV Env are required for transformation. Although the investigators do not point this out, it might turn out to be important that all exogenous JSRVs have an arginine residue after the tyrosine in YXXM, *i.e.* YRXM (Fig. 3.22). The mutated form of pCMV3ΔGP(NruIend) would have a sequence of YKXM, and this might account for the finding of Maeda *et al.* (2003). It would have been interesting to see if a different result could have been achieved if the motif had been changed to YRTM, corresponding to JSRV isolates of African origin. This is not likely though, in view of the reports that the YXXM is dispensable to JSRV Env transformation in DF-1 cells (Allen *et al.*, 2002) as well as rodent fibroblasts under the correct conditions (Liu *et al.*, 2003b).

To confirm that their findings pertaining to the lack of involvement of PI3K in JSRV transformation of NIH 3T3 cells was not due to lack of expression of their constructs in cells expressing Δp85, or to altered subcellular localisation, Maeda *et al.* (2003) HA-

tagged their constructs to make their Env products immunologically detectable. They found that all their constructs were expressed at similar efficiency, and that subcellular localisation was the same for wild-type and YXXM-mutated constructs. Maeda *et al.* (2003) further refer to results not shown in the publication. Firstly, no tyrosine phosphorylation of the JSRV Env protein could be detected [consistent with Liu *et al.* (2003b)]. Secondly, no binding interaction between JSRV Env and the p85 subunit of PI3K was detected. The investigators discuss the possibility that PI3K-independent activation of Akt could be mediated by pathways involving either the cytoplasmic tail of TM or other envelope protein domains including SU, despite the earlier report by Chow *et al.* (2003) that showed JSRV transformation not to require the surface domain. As an example of other motifs important to JSRV Env transformation, Maeda *et al.* (2003) refer to unpublished data of theirs – if a valine residue in the cytoplasmic tail near the membrane-spanning domain is mutated, transformation is abrogated. They do not identify the position of this residue, nor whether or not it is shared by enJSRVs.

#### 3.4.2.11 Hyal2 does not directly modulate RON activity in rodent fibroblasts

In an effort to further characterise the involvement of RON and Hyal2 in the JSRV Env mediated transformation of epithelial cells (Danilkovitch-Miagkova *et al.*, 2003), this group attempted to investigate these interactions in rodent fibroblasts (Miller *et al.*, 2004). They had encountered difficulties in reliably transfecting the BEAS-2B cells used in their previous study, and found the reproducibility of their transformation assays to be poor. Rodent fibroblasts are the most commonly used cell lines for transformation assays, and other reports had indicated that they can be transformed by Stk, the mouse orthologue of

RON. Danilkovitch-Miagkova *et al.* (2003) had concluded that Hyal2 down-regulates RON/Stk activity and that this down-regulation is inhibited by JSRV Env binding to Hyal2. Miller *et al.* (2004) reasoned that they should therefore be able to suppress transformation mediated via the RON/Stk pathway by overexpressing Hyal2.

Miller *et al.* (2004) transfected NIH 3T3 cells with plasmids expressing either RON or Stk (the human and rodent orthologues, respectively). As mentioned before, these cells do not naturally express this receptor. They found no transformation of NIH 3T3 cells as a result of RON or Stk expression. When repeating these experiments using rat 208F cells however, they did find extremely low levels of transformation (less than 1% of the transformation activity observed for JSRV Env). They established stably transformed cell lines from the transformants and treated them with human macrophage stimulating protein (MSP), the natural ligand of RON/Stk. As expected, they observed that the transformed phenotype of RON/Stk-transformed 208F cells was enhanced by treatment with MSP. Surprisingly, the transduction of the RON/Stk transformed cell lines (whether they expressed MSP or not) with human or rodent Hyal2 had no effect on their morphologies and phenotype. This shows that, contrary to the conclusions of their earlier study (Danilkovitch-Miagkova *et al.*, 2003), Hyal2 does not directly modulate the activity of RON/Stk, at least in this system that uses 208F cells. It therefore seems likely that additional factors are present in BEAS-2B cells that are required for Hyal2 regulation of RON activity. The investigators maintain that the RON pathway might be more important in JSRV oncogenesis *in vivo* than the alternative pathway(s) suggested for rodent fibroblasts, since the natural targets for JSRV transformation are lung epithelial cells.

### 3.4.2.12 Evidence for SU requirement in JSRV Env transformation

Despite the report by Chow *et al.* (2003) that the surface (SU) protein of JSRV Env is dispensable to JSRV transformation of rodent fibroblasts, Hofacre & Fan (2004) presented evidence that it is required. They created three sets of constructs mutated in SU. The first set had increasingly larger SU deletions, all starting downstream from the endoplasmic reticulum (ER) signal recognition peptide (SRP) so as not to affect trafficking to the cell membrane. Dirks *et al.* (2002) had suggested that the JSRV Env ER SRP lies upstream of residue 77, so all deletions had their 5' endpoint at residue 102, where a *Pst*I linker had been inserted for cloning purposes (Hofacre & Fan, 2004). None of these deletion constructs induced transformation of either NIH 3T3 or 208F cells, even the one with the smallest deletion of about 50 amino acids.

The second set of constructs tested by Hofacre & Fan (2004) for SU involvement in JSRV Env transformation had nested 50-amino acids deletions throughout SU (Fig. 3.26). None

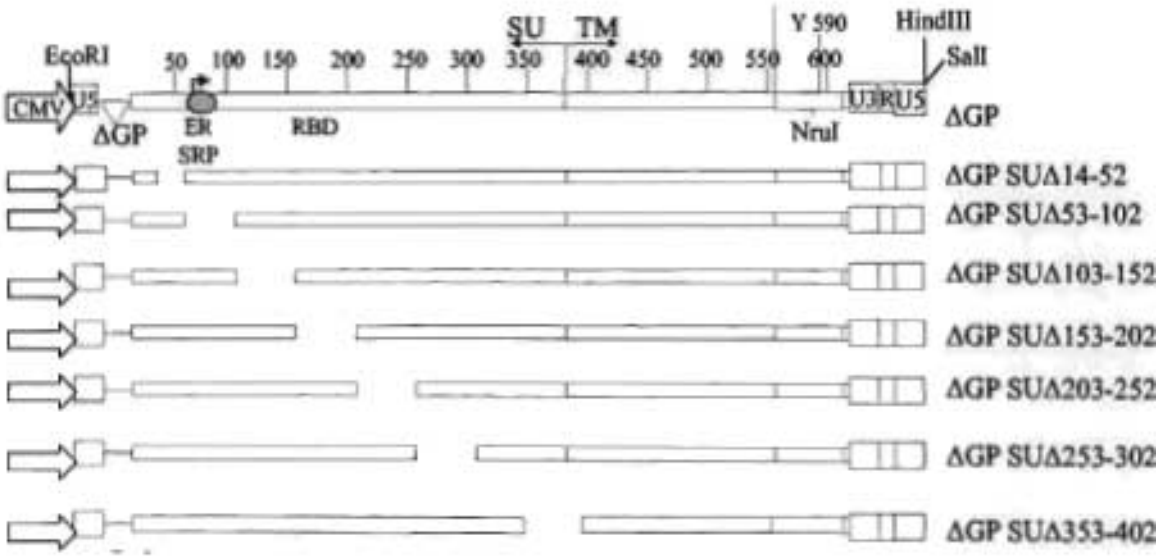



**Figure 3.26 JSRV Env expression constructs with nested 50-amino acid deletions throughout SU.** Adapted from Hofacre & Fan (2004).

of these transformed NIH 3T3 cells either. One of the constructs, however, was able to transform 208F cells. This construct (ΔGP SUΔ14-52) had its deletion upstream of the ER SRP. The third set of mutants comprised a set of minimal insertions of two amino acids at various points throughout SU. Once again, none of these minimal insertion mutants transformed NIH 3T3 cells, whereas one transformed 208F cells. This construct also had its two amino acid insertion upstream of the ER SRP, and the investigators presume that the reason the two constructs that are mutated upstream of the ER SRP are able to transform 208F cells is because this region would be cleaved off as part of the removal of the signal peptide in ER, thus forming wild-type SU. The investigators do not offer a suggestion as to why these two constructs do not transform NIH 3T3 cells if they transform 208F cells.

The above results indicate a need for the SU protein in JSRV transformation of rodent fibroblasts. Hofacre & Fan (2004) addressed possible alternative explanations of their results, firstly by verifying the reading frames of all their constructs and secondly, by showing that the mRNA expression of deletion constructs is not reduced relative to that of the full-length Env expression construct. Thirdly, they also HA-tagged several of their constructs to enable antibody detection of the products. This allowed them to demonstrate that the products derived from the deletion and minimal insertion products were equally stable as the wild-type JSRV Env, and that they were also correctly localised to the cell membrane.

Hofacre & Fan (2004) proposed that the JSRV Env SU and TM domains act independently to facilitate transformation. In support of this hypothesis, they conducted various co-transfections of 208F cells with pairwise combinations of SU- and TM-mutated constructs

that were each not able to induce transformation by themselves. These experiments were, of necessity, preceded by the identification of non-transforming TM mutants. Consistent with Liu *et al.* (2003b), they found that an Env expression construct with the methionine residue of the "critical" YXXM motif (Fig. 3.21 and 3.22) mutated, was able to induce transformation of 208F cells at decreased efficiency. Contrary to the findings of Liu *et al.* (2003b) however, in the hands of Hofacre & Fan (2004), mutation of the tyrosine residue (in a construct they call ΔGP Y590F) abolished transformation of 208F cells. They therefore used ΔGP Y590F (which would encode a functional SU) in co-transfections with three different SU-altered non-transforming constructs (which would each encode functional TM). In each of these co-transfection studies, the SU encoded by ΔGP Y590F complemented the TM encoded by the SU mutants to result in efficient transformation of 208F cells.

In the discussion of their results, Hofacre & Fan (2004) refer to the apparent inconsistency of their findings with those of Chow *et al.* (2003), who had shown SU to be dispensable to JSRV Env transformation. Hofacre & Fan (2004) argue that the deletion of the putative receptor binding domain of JSRV by Chow *et al.* (2003) left the carboxy-terminal portion of SU intact (Fig. 3.24B), and propose that this region might be important for transformation, in combination with the PI3K-independent activation of Akt, mediated by JSRV TM. They refer to unpublished data of theirs that indicate JSRV Env binding to Toll-like receptor 4 (TLR-4), and cite personal communication with another researcher who has found TLR-4 to bind to the carboxy-terminal portion of mouse mammary tumour virus SU. However, Hofacre & Fan (2004) apparently disregard two facts: Firstly, most of their SU deletion constructs also left the carboxy-terminal portion of SU intact (Fig 3.26), raising the question of why these were not sufficient to induce transformation; secondly, Chow *et*

*al.* (2003) also achieved efficient transformation of 208F cells with a construct that only expressed the carboxy-terminal 141 amino acids of JSRV Env (Fig. 3.25, pMyr-JSE6770). This construct would be missing all of SU, as well as the amino-terminal portion of TM. Both studies demonstrated correct localisation of their Env constructs to the cell membrane, so the fact that Chow *et al.* (2003) used a myristoylation signal and Hofacre & Fan (2004) relied on the ER SRP native to JSRV SU should make no difference.

#### 3.4.2.13 JSRV Env transformation of canine kidney epithelial cells

Up until this point, it appeared as though JSRV Env transformation *in vitro* operates via two entirely distinct mechanisms for fibroblasts and for epithelial cells (Liu & Miller, 2005). Since most studies had focussed on rodent fibroblasts, yet the natural targets for JSRV oncogenesis are epithelial cells, Liu & Miller (2005) once again used an epithelial cell system to study JSRV Env transformation. Owing to various difficulties experienced with the BEAS-2B cell line used in their earlier study involving epithelial cells (Danilkovitch-Miagkova *et al.*, 2003), they now turned to the Madin-Darby canine kidney (MDCK) epithelial cell line. Their expression constructs were once again based on pSX2neo, driven by an MLV promoter. Using these, they showed that JSRV and ENTV Env can transform MDCK cells, and that their respective cytoplasmic tails are essential for MDCK transformation. In agreement with their findings in 208F cells (Liu *et al.*, 2003b), the $Y^{590}$-X-X-$M^{593}$ motif is not required for transformation of MDCK cells, either by JSRV or by ENTV Env. In fact, in MDCK cells the transformation efficiencies for all mutations of the Tyr or Met residues were in excess of 20% compared to the wild-type Env-expressing constructs – a lot higher than in 208F cells.

Next, Liu & Miller (2005) showed that Akt was phosphorylated in all the Env-transformed MDCK cells (including those transformed by YXXM mutants), and that this Akt phosphorylation could be completely inhibited by LY294002. LY294002 treatment also partially reverted the morphology of transformed MDCK cells back to their non-transformed state. These results indicated a requirement for PI3K activation in Env transformation of these cells. However, Liu & Miller (2005) detected neither Env phosphorylation (Tyr phosphorylation in YXXM would be necessary for binding PI3K) nor coimmunoprecipitation of Env with the p85 regulatory subunit of PI3K, implying that JSRV and ENTV Env proteins activate PI3K/Akt through one or more unidentified adaptor molecules, as already suggested by Allan *et al.* (2002), working on chicken DF-1 cells. The same would then be true for 208F cells (Liu & Miller, 2005).

To complete the comparison of JSRV and ENTV Env transformation in BEAS-2B and MDCK cells, Liu & Miller (2005) showed that, in contrast with BEAS-2B cells, neither RON nor Hyal2 played a role in transformation of MDCK cells. (Interestingly, they observed that canine Hyal2 can function as a receptor for JSRV, but not for ENTV.) The investigators therefore conclude that the transformation mechanism operative in MDCK cells is likely to be the same as in 208F rat fibroblasts. In the discussion of their results, they mention that they were not able to transform MDCK cells with the same construct used by Chow *et al.* (2003) that expresses the carboxy-terminal 141 amino acids of JSRV TM (Fig. 3.25, pMyr-JSE7061), and that they only achieved minimal transformation of 208F cells with this construct, as opposed to Chow *et al.* (2003), in whose hands it supported efficient 208F transformation.

### 3.4.2.14 Summary and discussion

In summary, the available experimental evidence at the time when the author concluded his practical research work described in this thesis favours the existence of two alternative models to explain JSRV Env transformation *in vitro*. The first has thus far only been shown to apply to the immortalised non-transformed human bronchial epithelial cell line BEAS-2B (Fig. 3.23). This model proposes that Hyal2, the cellular receptor for JSRV and ENTV, normally functions to negatively regulate a receptor tyrosine kinase, RON. Infection with JSRV particles would be mediated by binding of the receptor binding domain (RBD) within the SU protein to Hyal2. JSRV replication would lead to Env expression in the infected cell and Env proteins would be trafficked to the cell membrane where they once again bind Hyal2, in competition with RON. The Env/Hyal2 complex is internalised and degraded, leaving RON to autophosphorylate and subsequently activate oncogenic pathways involving mitogen activated protein kinase (MAPK) and Akt. Although the RBD in the SU protein of JSRV Env would obviously be required by this model, the involvement of other regions within JSRV Env have not been investigated.

Evidence for the second model of JSRV Env transformation has been gathered from transformation studies in immortalised rodent and chicken fibroblasts, as well as primary (non-immortalised) chicken embryo fibroblasts and canine kidney epithelial cells. Taking into account the data inconsistencies arising from the use of various JSRV Env expression constructs and mutants, for the transformation of different cell lines, this model currently proposes the following: Regions within the cytoplasmic tail of JSRV Env (part of VR3), possibly in combination with other elements contained in the TM protein, indirectly activate the PI3K/Akt pathway, leading to oncogenesis. Although one study indicates that

PI3K is not involved in this process, all other available evidence indicates that it is. The one requirement of JSRV transformation that all studies contributing to this model agree on is that of the cytoplasmic tail in VR3, and the common signature of both models (and all studies, in all cell lines) is the activation of Akt kinase.

The ideal *in vitro* system for JSRV transformation studies would obviously be sheep type II pneumocytes with indefinite growth potential. Unfortunately, type II pneumocytes from any animal have proven very difficult to maintain in culture for any length of time, and they tend to lose their state of differentiation quickly (various personal communications). In fact, at the CRI/UCI the author was witness to numerous unsuccessful attempts at culturing these cells from rats, and establishing stable cell lines. However, the published abstract of a conference presentation (DeMartini *et al.*, 2004) reports the establishment of a primary sheep alveolar type II cell line that was successfully maintained in culture for seven to fourteen days and productively infected with JSRV. It will be most interesting to follow the outcomes of studies conducted with these cells. Further encouragement in this regard has been presented by the results of a recently published study (Archer *et al.*, 2007), where alveolar type II cells from OPA tumours were successfully established as cell lines. Two proliferative cell subpopulations were identified: The first expressed JSRV, but could not be maintained for more than seven passages. The second was a transformed, yet JSRV-negative population that displayed long-term maintenance *in vitro*. The investigators propose that the latter subpopulation is acutely transformed by JSRV Env in the absence of integrated JSRV, and express their intention of determining whether JSRV is detrimental to those cells that it infects.

Finally, whereas *in vitro* studies are of the utmost importance in helping to dissect the

potential mechanisms of transformation in a disease such as OPA, the aim is ultimately to determine each of the steps leading to the disease in sheep infected with JSRV. *In vivo* infection studies by different groups, using numerous mutated JSRV forms (e.g. Δ*orf-x*, Env Y590F, etc.) are currently underway (various personal communications), and it is very likely that differences between the *in vitro* models and the *in vivo* effects will be noted. These will then need to be investigated and explained using *in vitro* studies once again. Already at this point, it is not certain that the *in vitro* signature of JSRV transformation – Akt activation – is applicable to the disease situation, when considering that OPA tumours were not found to contain phosphorylated Akt. It is to be expected that the naturally occurring, as well as experimentally induced, disease progression will be far more complex than any simple model based purely on *in vitro* studies is likely to be able to explain.

# CHAPTER 4

# GENERAL DISCUSSION AND CONCLUSIONS

Ovine pulmonary adenocarcinoma (OPA) serves as a valuable, naturally occurring outbred animal model for peripheral human lung carcinomas, and to epithelial cancers generally, since most other animal models of retrovirally induced cancer pertain to the haematopoietic system. This study has significantly advanced the specificity and underlying comprehension pertaining to this model. This chapter summarises and discusses the major findings of the three aspects of the study described in this thesis. The first phase comprised the cloning and sequence determination of three full-length endogenous JSRV loci from sheep. Comparison with exogenous JSRV sequences led to the discovery of three variable regions in JSRV that consistently distinguish exogenous pathogenic from endogenous non-pathogenic JSRV isolates. These were termed the variable regions of JSRV: VR1 and VR2 are found in *gag*, whereas VR3 is located at the carboxy-terminus of *env*, comprising the 3' end of the transmembrane region and encompassing the cytoplasmic tail. The second phase of the study consisted of the author's pursuit of creating relevant biological constructs for the future study of the significance of VR1 and VR2. Concurrently, his international colleagues focussed their attention on VR3, and linked it to transformation *in vitro*. While the author did not have the opportunity of contributing any laboratory work to this third aspect of the study, he was intricately involved in its progressive development. Finally, this chapter will present overall conclusions and directions for the continuation of this work.

## 4.1 DISCOVERY OF VARIABLE REGIONS IN JSRV

The determination of the sequence of JSRV (York *et al.*, 1991; York *et al.*, 1992) was arguably the single greatest advancement in JSRV molecular biology research at the time of its publication, and saw in a new era in this field. The work has been cited 126 times in subsequent publications. Having the complete nucleotide sequence of JSRV at their disposal, however, appeared to bring researchers in the field no closer to identifying genetic elements or regions within the JSRV genome to implicate in its pathogenic role in OPA. In fact, until the results of this study were published, no further discoveries pertaining to JSRV-encoded pathogenesis were made. Given this long passage of time since the elucidation of the genomic sequence of JSRV, it was decided to resolve this impasse by conducting a detailed comparison of the sequences of exogenous pathogenic JSRV isolates with non-pathogenic endogenous ones. It was hypothesised that such a comparison would identify some elements or regions that consistently distinguish JSRV from enJSRVs. Such regions could then become the focus of further investigation.

A comprehensive comparison necessitates the use of several sequences in order to differentiate between mutations in enJSRVs accumulated since their integration into the sheep germ line and those differences with regard to JSRV that are common to enJSRVs in general. An initial approach to this was the PCR amplification of two sub-genomic enJSRV fragments that, taken together, would reconstitute almost an entire enJSRV locus. Given the multiplicity of enJSRVs in the sheep genome, however, there was no indication that the two sub-genomic fragments were from the same locus. A different approach was adopted, whereby a sheep genomic library was screened for full-length enJSRV loci. Three

of these were successfully sub-cloned and their full proviral sequences were determined. These constituted the first-ever full-length enJSRV genomes to be sequenced.

Analysis of the three full-length enJSRV sequences identified several characteristics that were unique to individual loci, as well as yielding novel insights into the evolution of phylogenetics of enJSRVs. More importantly, however, it led to the accomplishment of the goals that had been envisaged for this aspect of the study, *viz.* the discovery that JSRVs and enJSRVs are consistently distinguished from one another in three short regions within their coding genes. This was precisely the type of finding that the author and his colleagues, both locally and abroad, had envisaged in the design of the study. These three regions were named the variable regions of ovine betaretroviruses, and comprised VR1 and VR2 in the first two thirds of *gag*, and VR3 at the 3' terminus of *env*. The remainder of enJSRV genomes were found to be remarkably similar and homologous to JSRV isolates.

Given their specialised facilities and extensive expertise, the author's international colleagues at the CRI/UCI embarked on the investigation of VR3's role in transformation *in vitro*. This work was, from the outset, an integral, albeit non-laboratory based part of the author's own study. Concurrently, the author accepted the challenge of developing molecular biological tools that could be used, down the line, to discern the significance of VR1 and VR2.

## 4.2 VR3 AND JSRV ENVELOPE TRANSFORMATION

The author did not have the opportunity to conduct laboratory-based research into the role of VR3 in JSRV pathogenesis, since our laboratory did not have the relevant facilities for the associated studies. It was anticipated that any involvement of VR3 in transformation could be more readily detected *in vitro* than the effects of VR1 and VR2, since the envelope protein interacts with cell surface receptors and has more opportunity of altering cellular functions, the resulting phenotype whereof can be observed in tissue culture. In an effort to maintain an overall perspective of the progress made in JSRV pathogenesis research, the author was required to keep abreast of the work being undertaken in this field, both by our collaborators and by other investigators. This was done by corresponding with various laboratories involved in JSRV transformation research, and by critically appraising the work being published, and by providing feedback to our colleagues. The *in vitro* transformation studies produced a tremendous amount of data during the time that the author was conducting his laboratory-based research involving VR1 and VR2, and it is to be borne in mind that the development of this entire field was based on the earlier findings of this study, as already discussed.

The fact that human cells express the JSRV receptor HYAL2 and are transduced by JSRV (Rai *et al.*, 2000), taken together with the finding that 30% of human bronchioloalveolar carcinoma and pulmonary adenocarcinoma samples, but not adenocarcinomas from other organs, are immunopositive using antibodies against the JSRV capsid protein (De las Heras *et al.*, 2000), raises justified concerns that JSRV or a related virus might be carcinogenic in humans. Such fears were allayed to some degree by a report that showed immunodeficient mice to be susceptible to the development of JSRV Env-induced

bronchioloalveolar tumours upon infection with an adeno-associated virus vector expressing JSRV Env (Wootton *et al.*, 2005), while immunocompetent mice were resistant to tumour development. Since mice do not express Hyal2 that is functional as a JSRV receptor, tumourigenesis occurred in the absence of Hyal2-mediated cell entry. The investigators conclude that sheep are likely to be susceptible to JSRV transformation owing to the expression of enJSRVs and resultant immunotolerance towards JSRV. Since enJSRVs are absent in other species, their immune recognition of JSRV Env is expected to protect against JSRV tumourigenesis (Wootton *et al.*, 2005).

In drawing their conclusions, (Wootton *et al.*, 2005) appear to be unaware of an earlier study that describes the finding of JSRV *orf-x* sequences in 19 of 64 and *gag-pro* sequences in 4 of 38 samples, mainly from individuals from Cameroon and Nigeria (Morozov *et al.*, 2004). The investigators excluded the possibility that the JSRV sequences they detected in these samples were food-derived by demonstrating an absence of sheep- or goat-specific mitochondrial DNA. They did not establish a link with lung cancer (this had been the objective of their study), but they noted that they detected JSRV sequences more frequently in HIV-patients than in healthy blood donors. They suggest that a JSRV-like virus might circulate among people from some parts of Africa, and that AIDS predisposes to infection with this virus. However, the fact that JSRV sequences were detected in healthy controls implies that they were infected with a JSRV-like virus. This does not contradict the immune protection afforded against JSRV-induced tumourigenesis suggested by (Wootton *et al.*, 2005). Considering the high prevalence of HIV and associated immunodeficiency, the possibility of human infection with JSRV, and subsequent tumourigenesis remains a concern in the author's opinion.

The use of JSRV Env to pseudotype feline immunodeficiency (FIV)-based lentiviral vectors constructed for the transduction of airway epithelia has recently been reported (Sinn *et al.*, 2005b). This study followed an earlier report that the inclusion of JSRV proviral sequences (*env* and some of its flanking regions) in these lentiviral vectors led to a 10,000-fold increase in titre (Sinn *et al.*, 2005a). In their discussion, (Sinn *et al.*, 2005b) refer to unpublished results that show a Y590D mutation of JSRV Env to lead to a dramatic drop in FIV pseudotyping efficiency. The mutation had been introduced to address concerns regarding JSRV Env's oncogenic potential, which would constitute a problem if JSRV Env were to be considered as a pseudotyping candidate for gene transfer. The investigators express hope that the presentation of JSRV Env in the context of a replication incompetent retroviral vector might not have any harmful effects. The creation of a JSRV-based replication-defective virus expressing JSRV *env* under the control of its own LTR has recently been described (Caporale *et al.*, 2006), and was shown to induce OPA in newborn lambs. The implication of these findings is that JSRV viral spread after infection is not required to induce OPA.

A recent study was successful in developing mouse monoclonal antibodies that very specifically recognise JSRV Env in the tumours of naturally OPA-affected sheep from Europe, USA and Africa, as well as in JSRV Env-induced tumours in mice (Wootton *et al.*, 2006). These antibodies could be used in the context of a highly specific diagnostic test for OPA, and possibly for ENTV. Other observations in this study were that Env expression is limited to tumours, and the investigators propose that JSRV Env might be toxic to the majority of differentiated lung cell types in sheep, accounting for the relative lack of observed metastasis. The final conclusion of this study is that the primary effect of JSRV infection is to drive localised proliferation of lung epithelial cells, which contrasts with the

findings of (Caporale *et al.*, 2005), namely that infection of lung epithelial cells and induction of OPA is not the most common outcome of JSRV infection.

It is evident from the above selection of recent studies on JSRV transformation and Env that this is an emerging field, attracting ever-increasing numbers of investigators. It presents an array of interesting challenges that, if met, will provide profound insights into an oncogenic retrovirus that is gradually becoming more understood.

## 4.3 FUNCTIONAL ANALYSIS OF VR1- AND VR2-SUBSTITUTED CONSTRUCTS

Some might be tempted to argue that the involvement of the JSRV envelope protein in transformation, in the absence of *gag*, diminishes the importance of investigating VR1 and VR2 in any great detail. However, there are compelling scientific reasons to investigate the two variable regions in *gag*. They, together with only one other conserved variable region, clearly distinguish an exogenous retrovirus that causes an infectious ovine lung cancer from 15 to 20 very closely related endogenous proviral loci that reside benignly in the genetic makeup of sheep. It is expected that the effects of VR1 and VR2 will be subtle and that the biological significance of these variable regions will most likely only be discernible *in vivo*. Such tests will form the basis of another study. It was the aim of this study to develop the molecular tools that would, in future, be used to test the functions of VR1 and VR2, and to characterise these tools *in vitro* to establish their suitability for further *in vivo* studies.

This final objective of the study described in this thesis was met by precisely substituting VR1 and VR2, individually and in combination, into the exogenous infectious molecular clone pCMV2JS$_{21}$. The strategy developed for this precise substitution was novel, and in spite of its conceptual complexity, was shown to be implemented with relative ease once the experimental pitfalls had been identified. The method was superior in its specificity compared to more conventional approaches, as was demonstrated by the fact that a parallel experiment carried out by another group culminated in the presentation of inaccurate conclusions regarding the involvement of VR1 in the lack of enJSRV particle formation. Furthermore, it was shown that this substitution strategy is readily adaptable to the substitution of any PCR-amplifiable region, even between entirely unrelated DNA sequences. It is therefore a highly versatile tool with broad applications in molecular biology.

The objectives of this last aspect of the study were met, in that the tools were developed that could be used to test the biological significance of VR1 and VR2. These tools comprise the three chimeric constructs pJS$_{21}$/56A1VR1, pJS$_{21}$/56A1VR2 and pJS$_{21}$/56A1VR1VR2. A prerequisite for the progression to *in vivo* testing of these constructs was that at least some of them support retroviral particle formation, as this is the form in which they will need to be administered. It was shown that all three chimeric constructs supported abundant particle formation *in vitro*, with levels comparable to the non-substituted wild-type pCMV2JS$_{21}$ in the case of pJS$_{21}$/56A1VR1, and exceeding it in the case of pJS$_{21}$/56A1VR2 and pJS$_{21}$/56A1VR1VR2. A further condition for the use of the chimeric retroviral particles *in vivo* is that they be able to infect target/host cells and integrate their genomes into those of the infected cells. This was shown to be the case by

use of *in vitro* infectivity assays, thereby rendering the chimeric constructs fully qualified for *in vivo* tests to ascertain the significance of VR1 and VR2.

## 4.4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion, all aims and objectives set out at the beginning of the various stages of this project were met. The first three (and so far only) full-length enJSRV proviral sequences were determined. These were comprehensively analysed, and three variable regions were identified that consistently differentiate exogenous pathogenic from endogenous non-pathogenic JSRV isolates. One of these variable regions has already been successfully linked to JSRV-induced transformation, both *in vitro* and *in vivo*. Finally, the molecular tools were generated that will be used to elucidate the more subtle biological effects of the other two variable regions, and the suitability of these tools for future studies has been validated.

This work has already had a broad impact in the field of JSRV research, with the earliest publication arising from it having been cited 38 times so far in other ISI-recognised journals. The identification of variable regions specific to exogenous JSRVs has led to the design of new diagnostic tests that exceed the specificity of previously used tests (Padayachi, 2005). However, this work did not have a veterinary focus, and these benefits are secondary. More importantly, the work has led to profound progress in furthering the understanding of OPA as a uniquely useful model of human disease.

Future directions leading on directly from this work will include the *in vivo* testing of the particles derived from the three chimeric constructs developed in this study. It will be most interesting to observe the effects of endogenous VR1 and VR2 in the context of the exogenous JSRV. Also, reciprocal constructs will be created using the novel substitution strategy developed in this study, based on a combination of the sequences of the three endogenous proviruses. The point mutations that have been identified as being inhibitory to particle release in enJS56A1 will either be avoided by using *gag* from one of the other enJSRVs, or corrected by site-directed mutagenesis. Exogenous VR1 and VR2 will be substituted into the constructs, providing an endogenous backbone with exogenous VR1 and VR2.

# CHAPTER 5

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# APPENDIX I

GenBank accession numbers of sequences arising from, referred to in, or pertinent to, this thesis:

| | |
|---|---|
| enJS56A1 | AF153615 |
| enJS5F16 | AF136224 |
| enJS59A1 | AF136225 |
| ENTV | Y16627 |
| ENTV-2 | AY197548 |
| JSRV-SA | M80216 |
| JSRV21 | AF105220 |
| JSRVJS7 | AF357971 |
| caenENTVgag | AY372190 |
| ESRVLTRL2 | X95446 |
| caERV2U3 | AY196353 |
| caERVU3 | AY196352 |
| TNC196eL | AY196356 |
| TNC396eL | AY196355 |
| TNC294eL | AY196354 |
| TNO35eL | AY196359 |
| oENTVenvPrec | AF401741 |
| TNO28eL | AY196357 |
| TNO29eL | AY196358 |
| ESRVLTRL6 | X95450 |
| 92K3 | Y18305 |
| ESRVLTRL5 | X95449 |
| ESRVLTRL1 | X95445 |
| ESRVLTRL4 | X95448 |
| ESRVLTRL3 | X95447 |
| enJRVLTR2 | Z66532 |
| enJRVLTR1 | Z66531 |
| 809T | Y18302 |
| 84R528 | Y18304 |
| 83R528 | Y18303 |
| enJRVLTR3 | Z66533 |
| exLTR_UK | Z71304 |
| exLTRt2 | X95452 |
| exLTRt1 | X95451 |
| EN54 | Y18307 |
| Y16 | Y18306 |
| enJRVGAG2 | X92180 |
| enJRVGAG1 | Z49800 |
| OPVCAPD | L29127 |
| enJRVGAG4 | X92182 |
| enJRVGAG3 | X92181 |
| oENTV envU3 | AY271312 |

# APPENDIX II

Sequences of DNA oligonucleotide primers designed specifically for use in this study:

| Position in $JSRV_{21}$ | Sequence (5' – 3') |
|---|---|
| 35R | TTGAGAGGATCAGCCCAAAATGGCTGATAC |
| 124F | GCAGGTGGCGCCCAACGT |
| 481F | AAGTTCCTGTGGAAACTTTATCC |
| 533F | CTTTGATAATGATGAATTAAAACGTTTAGG |
| 568F | TATTAAAGCAGGAAGAAGATCCTCTTCAT |
| 596R | ATGAAGAGGATCTTCTTCCTGC |
| 660F | CCTTCAGACAATGATGATTTACTTTC |
| 688R | GAAGAAAGTAAATCATCATTGTCTGAAGG |
| 810F | CTTACTATTGCTTTACAAAATGCAGGAAT |
| 838R | ATTCCTGCATTTTGTAAAGCAATAGTAAG |
| 954F | ATGTCTCCCCTACAAAGGGCC |
| 976R | AAGGCCCTTTGTAGGGGAGACAT |
| 1038R | CAAAAACAGGAAAGGCAAGAG |
| 1157R | CAAATTTTCTATCATAGCAATGGTGAATGG |
| 1396R | GCCGCATTTGATATTTGTGCA |
| 1608R | GTCTTAAAGCAGCTTGACAA |
| 6748F | TTATAAATGGATTTGTGTTACAAAAAAGCC |