ONCOGENIC PROGRESSION IN RETROVIRUS-INDUCED T-CELL LEUKEMIA

by

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STATEMENT

The experiments presented in this thesis represent my own work, unless otherwise acknowledged. Some of this work has been accepted for publication. This manuscript has not been previously submitted for a degree at this or any other university.

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ABSTRACT

T cell leukemogenesis induced by slowly transforming retroviruses is a multistep process associated with a long latency period and clonal selection within the thymus. To circumvent the long time required for leukemia development, a Radiation leukemia virus (RadLV)-infected cell line, C1-V13D, was investigated as a model for studying the oncogenic process. This cell line was tested and shown to undergo progressive transformation when passaged serially through the thymus, allowing at each step a dissection of event(s) associated with multi-step carcinogenesis. On this basis, C1-V13D was used as a model to elucidate the mechanism(s) contributing to oncogenic progression in T cell leukemia induced by retroviruses.

Investigations into changes in C1-V13D during intrathymic replication indicated that this cell line was capable of differentiation and expression of T cell surface antigens. This result supported several lines of evidence that progression in leukemia was often associated with a shift towards a more mature cell phenotype, despite the fact that the disease usually originated in an immature or stem cell population. Genetic changes associated with differentiation of C1-V13D within the thymus may have involved proto-oncogenes associated with cellular growth and differentiation.

Further retroviral events have been associated with the oncogenic process, either through generation of more leukemogenic viruses by recombination, or through disruption of cellular genes via proviral integration. Studies undertaken to determine if further retroviral events had occurred in C1-V13D indicated that these events did not contribute to the emergence of progressively tumorigenic C1-V13D at each intrathymic passage.

Characterisation of cellular genes in the vicinity of retroviral integration sites in C1-V13D was initiated with the objective of uncovering novel proto-oncogenes which may play a role in the transformation of precursor lymphoid cells. Several proviruses were found to be integrated into regions of DNA carrying repeat elements.

Some of these elements have been documented to encode proteins or have regulatory roles. In addition, they have been associated with many cancers as a result of their ability to retrotranspose. The frequent presence of repeat elements in the vicinity of proviral integration indicated that retroviral insertions in C1-V13D may not be completely random. Repeat elements may play a significant role in cell transformation or oncogenic progression.

A mouse flanking sequence adjacent to the 5'LTR in a provirus cloned from C1-V13D was also characterised. Preliminary investigation has indicated that the expression of this gene is tissue specific in the adult mouse. Computational analysis revealed that this flanking sequence could encode a stretch of charged amino acid residues. Similar stretches of charged amino acids have been associated with proteins with regulatory functions. The cloning of a cDNA corresponding to this region from C1-V13D is proposed as a future strategy for more definitive characterisation.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
ddH20	deionised distilled water
DEPC	diethylpyrocarbonate
dNTP	deoxynucleoside 5' triphosphate
ddNTP	dideoxynucleoside 5' triphosphate
dGTP	deoxyguanosine 5' triphosphate
dATP	deoxyadenosine 5' triphosphate
dCTP	deoxycytidine 5' triphosphate
dTTP	deoxythymidine 5' triphosphate
DTT	dithiothreitol
ETDA	ethylene diamine tetra-acetic acid, disodium salt
g	relative centrifugal force
L	litre
M	molar
µg	microgram
mg	milligram
µl	microlitre
ml	millilitre
mM	millimolar
µM	micromolar
MOPS	morpholinopropanesulfonic acid
pg	picograms
pM	picomolar
rpm	revolutions per minute
SDS	sodium dodecyl sulphate

CHAPTER 1

General Introduction

1.1 Discovery of the murine leukemia viruses

It was only in 1951 that the viral etiology of leukemia was established by Ludwick Gross. He successfully induced leukemia in newborn C3H mice by inoculating them with cell-free extracts of tumour tissues from Ak mice (Gross, 1951). The murine leukemia viruses (MuLVs) are now described as a large family of replication competent retroviruses endogenous to laboratory and wild mice. Leukemia induced by these viruses involves complex interactions between the virus and host which is dependent on both the host genetic constitution and the nature of the virus (Tsichlis & Lazo, 1991).

1.2 Leukemia viruses of the mouse

Following the isolation of a leukemogenic virus responsible for spontaneous leukemia in an Ak mouse (subsequently designated the Passage A virus) (Gross, 1957), a number of other MuLVs were also isolated (Schoolman *et al.*, 1957; Friend, 1957; Graffi, 1958; Moloney, 1960; Lieberman & Kaplan, 1959). Some of these, in particular the pathogenic recombinant MCF (Mink cell focus forming) viruses (Hartley *et al.*, 1977; Chattopadhyay *et al.*, 1982) and the defective BM5d murine acquired immunodeficiency (MAIDS) virus (Chattopadhyay *et al.*, 1991), have been used extensively to study the mechanisms underlying the onset and development of murine leukemia.

Many attempts have been made to establish the relationship between various MuLVs on the basis of host range, the type of leukemia induced and host immunological responses (Gross, 1964; Moloney, 1962; Rich *et al.*, 1965;

Old & Boyse, 1965; Kaplan, 1967; Decleve *et al.*, 1976, 1977 & 1978). Further studies have also been carried out to compare viral genomes by restriction endonuclease mapping and sequencing (Rassart & Jolicoeur, 1980; Shinnick *et al.*, 1981; Chattopadhyay *et al.*, 1982; Grymes *et al.*, 1983; Wolff *et al.*, 1983; Bestwick *et al.*, 1984; Aziz *et al.*, 1989; Poliquin *et al.*, 1992). Differences in leukemogenic potential and tissue/cell tropism between MuLVs and their variants have now been mapped to specific viral determinants like the LTR, *env*, *gag* or *gag-pol* genes (Lenz *et al.*, 1982; DesGroseillers *et al.*, 1983 & 1984; Villemur *et al.*, 1983; Janowski *et al.*, 1985; Rassart *et al.*, 1988; Jolicoeur, 1991).

This review will concentrate on murine T cell leukemogenesis induced by non-acute type C retroviruses. However, the issues discussed in the following sections, such as target cells for MuLV infection, chromosomal abnormalities and insertional mutagenesis will also relate to other murine leukemias and to other types of retrovirally-induced cancers. These have been included for discussion where necessary to illustrate some of the general concepts and principles relating to retroviral pathogenesis.

1.3 Models for studying leukemogenesis and oncogenic progression

1.3.1 MuLVs as models

Murine leukemia bears many clinical and morphological similarities to leukemia observed in humans (Furth *et al.*, 1935; Gross, 1983). As a result, MuLVs have been used widely as convenient models to elucidate the mechanism(s) underlying the development of spontaneous leukemia (Dofuku, 1975; O'Donnell *et al.*, 1984; Hays *et al.*, 1989 & 1990). MuLV models have also been useful in the study of tumour induction and progression (Haran-Ghera, 1976; Haas *et al.*, 1984; Tsichlis *et al.*, 1985; Storch *et al.*, 1985; Davis *et al.*, 1987; Klinken *et al.*, 1988; Bergeron *et al.*, 1991). Non-oncogene bearing type C murine retroviruses such as the Moloney murine leukemia (MoMuLV) virus, which induce T cell leukemia, have been used extensively to identify novel cellular oncogenes involved in the different stages of the

tumorigenic process (Cuypers *et al.*, 1986; Breuer *et al.*, 1989; Bear *et al.*, 1989; Tsichlis *et al.*, 1990).

The Radiation leukemia viruses (RadLVs) are also type C retroviruses which carry no host sequences or oncogenes. Isolates of RadLV have been used to infect and immortalise primary spleen cells *in vitro* (O'Neill, 1992). One cell line established using this procedure has been investigated here as a model for studying T cell leukemogenesis. The use of RadLV as the leukemogenic agent could provide a model for studying leukemogenesis since RadLV-induced leukemia resembles poorly diffuse human lymphocytic lymphoma in its generalised form. It also mirrors primary mediastinal lymphoma of childhood, in which massive enlargement of the thymus and its invasion of the mediastinum causes death prior to generalized dissemination (Kaplan, 1977). The marked thymotropism of RadLV has indicated an important role for the thymus in the development of T cell leukemia (Miller, 1959; Haran-Ghera, 1966). Efficient replication of RadLV in immature thymocytes also makes this virus a useful tool for the study of intrathymic T-cell differentiation (Boniver *et al.*, 1989).

The RadLVs are ecotropic viruses, which means that they can only replicate in the species of origin. They were first isolated from thymic tumours induced in C57BL/Ka strain mice following split dose whole body irradiation (Lieberman & Kaplan, 1959). The inconsistent presence of these viruses in radiation-induced lymphoma cells has challenged their involvement in the disease (Ihle *et al.*, 1976; Lieberman *et al.*, 1979; Sankar-Mistry & Jolicoeur, 1980; Yefenof *et al.*, 1980; Rassart *et al.*, 1983; Janowski *et al.*, 1986). Despite the possible lack of a common etiology, both RadLV and x-ray irradiation have been observed to produce morphologically similar thymic lymphomas after a lengthy process of clonal selection in the thymus (Kaplan, 1967; Yefenof *et al.*, 1991; Kothler *et al.*, 1994). Leukemogenic isolates of RadLV have now been cloned from the BL/VL3 cell line, which was established by Lieberman and colleagues (1979) from a RadLV-induced thymic tumour. They have been extensively characterised and found to be distinctly different from other MuLVs and from other non-leukemogenic type C viruses endogenous to C57BL mice (Decleve *et al.*, 1976

& 1978; Manteuil-Brutlag *et al.*, 1980; Grymes *et al.*, 1983; Janowski *et al.*, 1985).

1.3.2 Use of special mouse strains

Nude mice and severely immuno-deficient *scid* mice have also proved useful as *in vivo* models for studying some human leukemias. Kamel-Reid *et al.* (1989) demonstrated that disease development following injection of a human lymphoblastic leukemic cell line into *scid* mice was similar to that seen in children. This model could be used for studying disease progression and for testing new treatment strategies. Recently, transgenic mice have been used to study the mechanisms underlying disease progression and to devise new therapeutic approaches to disease treatment. For example, Metcalf and Rasko (1993) used GM-CSF (Granulocyte macrophage - colony stimulating factor) transgenic mice to study the effects of GM-CSF on the FDC-P1 cell line, which is GM-CSF dependent. When inoculated with the GM-CSF dependent FDC-P1 cells, these GM-CSF producing transgenic mice die within 3 months from FDC-P1 overload in the lungs, liver, kidney, lymph nodes and spleen. This model lends itself to a re-evaluation of sustained GM-CSF administration to myelodysplastic patients with abnormal, potentially preleukemic granulocyte-macrophage populations. In cases like this, GM-CSF administration could lead to death, from accumulated pre-transformed or fully transformed leukemic cells.

1.3.3 Cell lines as models

The establishment of cell lines from tumours as experimental 'tools' has been valuable in the study of the oncogenic process (Haas & Meshorer, 1979; Schwartz *et al.*, 1986; Metcalf & Nicola, 1991; Zhang *et al.*, 1993). Thymic epithelial or bone marrow culture systems have been used to study cellular interactions that are essential for both normal and tumour lines, as well as spontaneous transformation *in vitro* (Nishi *et al.*, 1982; Naparstek *et al.*, 1986; Ogawa *et al.*, 1989). Using retroviral constructs carrying oncogene(s) to infect hematopoietic cells *in vitro*, it has been possible to investigate the mechanisms by which oncogenes mediate growth and differentiation of different hematopoietic cell types (Schwartz *et al.*, 1986; Stapleton *et al.*, 1991; Bonham *et al.*, 1992; Chisholm & Symonds,

1992; Miller *et al.*, 1992). By using cell lines as models, it has also been possible to demonstrate that growth factor independence *in vitro* is often parallelled by tumorigenicity development *in vivo*. Studies have indicated that this process is controlled by oncogenes (Sachs, 1978; Heldin & Westermark, 1984; Gordon *et al.*, 1985; Heard *et al.*, 1986; Ikeda *et al.*, 1993).

The primary advantage of using cell lines as models for studying tumorigenicity is that it is possible to perform experiments under conditions defined by the investigator. Factors involved in multistep progression to neoplasia can be dissected and studied, either alone or in combination, under well defined conditions. While the study of oncogenes using cell lines may not exactly parallel neoplastic transformation *in vivo*, it does provide a convenient system which can be used to confirm events occurring *in vivo*. Since leukemogenesis involves a multitude of events, it is not hard to justify the use of cell line models, in the hope of analysing just one or a few steps in this complex process.

1.4 Cells susceptible to infection and neoplastic transformation

The identification of the primary target cell type in many human leukemias is important in terms of disease association, prognosis and effective therapy (Campana *et al.*, 1987; Bassan *et al.*, 1992; Meng-er *et al.*, 1988). An analysis of human lymphoid leukemia cells by Greaves (1982) using a panel of antibodies revealed that the majority of these cells closely resemble lymphoid precursor or progenitor cells. This supports the hypothesis that most leukemias originate from expansion of immature lymphoid or stem cells.

Similar efforts have also been made to characterise the cell type susceptible to infection and initial neoplastic transformation in murine leukemias. Immature T cell precursors have been found to be preferred targets for MoMuLV transformation (Storch *et al.*, 1985; Lazo *et al.*, 1990), while lymphomagenic MCF viruses have been reported to selectively replicate in rapidly dividing immature lymphocytes present in the thymic cortex (Cloyd, 1983; O' Donnell *et al.*, 1984). In the case of RadLV-induced

leukemia, many studies were also conducted to localise the site of initial transformation. The results remained controversial. Some groups have found potentially neoplastic cells (preleukemic cells) in the bone marrow (Haran-Ghera, 1980; Naparstek *et al.*, 1986) while others have reported the presence of such cells in the thymus (Lieberman & Kaplan, 1966; Boniver *et al.*, 1981; Cloyd *et al.*, 1983).

Studies carried out to identify target cells for RadLV infection have relied upon light and/or electron microscopy (Haran-Ghera *et al.*, 1966 & 1979; Carnes *et al.*, 1968; Decleve *et al.*, 1974 & 1975), cell selection or depletion methods (Cloyd *et al.*, 1983) and transplantation assays, in which RadLV-infected or X-ray-irradiated cells from the thymus or bone marrow were inoculated into susceptible hosts (Lieberman & Kaplan, 1966; Kaplan & Lieberman, 1976; Boniver *et al.*, 1981). *In situ* studies on the location of infected cells, as well as transplantability of infected cells derived from the thymus and bone marrow have shown that although RadLV is capable of infecting many cell types in the fetal liver, lymph node, bone marrow, spleen and thymus, active viral replication only occurs in thymocytes which are at the earliest stage of T cell differentiation (Lieberman & Kaplan, 1976a). Recent data obtained from *in situ* hybridization experiments to detect viral transcripts has confirmed the susceptibility of the most immature thymocytes to RadLV infection (Boniver *et al.*, 1989).

An increasing number of reports have documented instances in which leukemic cells from both human and mouse co-express markers normally restricted to a single cell lineage (Smith *et al.*, 1983; Ha *et al.*, 1984; Palumbo *et al.*, 1984; Holmes *et al.*, 1986; Bassan *et al.*, 1992; Bonham *et al.*, 1992; O'Neill, 1992; Defresne *et al.*, 1993). This phenomenon has been termed 'lineage infidelity' to reflect altered gene expression brought about by genetic misprogramming (Palumbo *et al.*, 1984). This concept contrasted with studies by Greaves (1982) who used a panel of antibodies to demonstrate that leukemic cells were faithful with respect to cell lineage. The asynchrony of maturation-linked phenotype frequently observed in leukemic cells was proposed to reflect instead 'lineage promiscuity'. This was attributed to transient-phase bipotential or multipotential progenitors, simultaneously expressing different lineage-related or unrelated markers, under maturation arrest (Greaves *et al.*, 1986a). This alternative

explanation lends support to earlier evidence that immature or stem cells are the primary target cells for initial leukemic transformation.

1.5 Oncogenic progression in T-cell leukemogenesis

1.5.1 Preleukemic cell trafficking: The role of the thymic microenvironment

The need for an intact thymus during development of spontaneous leukemia in AKR mice and in RadLV-induced T cell leukemia was reported by Metcalf (1962) and Kaplan & Lieberman (1976), respectively. This was consistent with earlier studies which demonstrated that thymectomy substantially reduced the development of leukemia (Kaplan, 1950; Miller, 1959) and that susceptibility to the disease could be restored by thymic grafts (Kunii *et al.*, 1966). These studies do not exclude the possibility that cells could be infected elsewhere and subsequently migrate to the thymus for further transformation and disease progression (Asjo *et al.*, 1985). While a variety of cell types are susceptible to infection, the strict tropism of RadLV for thymocytes, and the formation of exclusively thymic lymphomas by RadLV, suggest a unique thymic dependence. This dependence could have arisen from a combination of factors including viral tropism, the nature of the target cell and the specific interaction of RadLV and RadLV-infected cells with thymic stroma or cellular elements. Thymic cellular elements have in fact been shown to stimulate the proliferation of leukemic T cell lines (Pinto & Rock, 1991) and to transform thymocytes *in vitro* in both the AKR and RadLV systems (Waksal, 1976; Haas *et al.*, 1977, respectively). A close symbiotic relationship has also been demonstrated between neoplastic lymphoid cells, specialised thymic nurse cells (TNCs) and stromal cell components (Hiai *et al.*, 1981).

These studies highlight the complexity of the leukemic process and accord with the view that the thymus plays a dominant role in T cell leukemogenesis. The thymus could allow transmission of leukemogenic viruses to susceptible thymocytes, produce factors needed for cell and viral replication (Schnittman *et al.*, 1991) or it could act as a safe, nurturing haven for preleukemic cells to survive, proliferate and

differentiate in the absence of a strong immune response (Werkele & Ketelsen, 1980; Nishi *et al.*, 1982; Ezaki *et al.*, 1991). The unique role of the thymus as an organ in which migrating, preleukemic hematopoietic cells can differentiate and progress to overt neoplasia is discussed in the following section.

1.5.2 Differentiation as a mechanism for oncogenic progression

Non-acute murine type C retroviruses have been reported to influence cellular differentiation by modulating or modifying the response of infected cells to normal regulators (Lieberman & Sachs, 1977). The murine leukemia viruses Moloney, MCF and RadLV produce T cell leukemias which reflect more mature T cell phenotypes and show phenotypic heterogeneity indicative of different stages of T cell differentiation (Lieberman *et al.*, 1979; O'Donnell *et al.*, 1984; Yefenof *et al.*, 1991). Two explanations are possible: a) T cells at all stages of development are susceptible to infection and transformation by these viruses or (b) these viruses transform immature T-cell progenitors, which then undergo further (possibly selective) differentiation in the thymus during oncogenic progression.

Numerous laboratories have produced indirect evidence supporting differentiation as an event intrinsic to the oncogenic process. For example, O'Donnell and colleagues (1984) demonstrated that during leukemogenesis, one of the changes occurring in the late preleukemic period in AKR mice was the shift to a more mature phenotype. A few independent but related studies on intrathymic cellular interactions have also supported this observation. One example is the remarkable increase in the number of TNCs before or at the time of onset of spontaneous thymomas in BUF/Mna rats (Ezaki *et al.*, 1991). These TNC populations were found to be hyperactive in their number and nursing capacity, resulting in an unusual increase in the number of Thy-1$^+$ CD8$^+$ CD4$^+$ thymocytes. TNCs have been shown to be directly involved in T cell differentiation and maturation (Kyewski & Kaplan, 1982; De Waal Malefijt *et al.*, 1986), and the presence of immature thymocytes within TNCs therefore relates to a critical stage of T cell differentiation. Using RadLV as a model, it was also

observed that the first RadLV-producing cells and the first leukemic cells were both located preferentially within TNCs (Goffinet *et al.*, 1983). These were identified using the marker for alkaline phosphatase. This is an enzyme expressed by cells at an early stage of T cell differentiation, such as lymphoblasts of the fetal thymus and lymphoid blast cells in close association with TNCs in the adult thymus. This data implied that the target cell population must undergo a critical differentiation step within the thymus in the natural progression to T cell leukemia. Earlier observations that immature cells are targets for infection and initial transformation (reviewed in Section 1.4), and that tumour cells continue to differentiate *in vitro* and *in vivo* (Green *et al.*, 1985; Lazo *et al.*, 1990) are consistent with the view that differentiation in the thymus is an important event in the progression of T cell leukemogenesis.

Recent evidence directly linking maturation events, increased tumorigenicity and disease progression was provided by Ishii and colleagues (1993) who reported the expression of CD10 by a leukemia cell line at the time of onset of dissemination of leukemia, using FACS analysis and PCR amplification. This correlated with progression of disease in *scid* mice. CD10 plays an undefined role in normal and malignant pre-B cell proliferation and differentiation (Law & Clark, 1994; Rosenberg *et al.*, 1994) and is not expressed on pro-B cells. It is, however, expressed by pre-B cells. The induction of CD10 could therefore indicate a further step along the differentiation pathway. Further supporting evidence was provided by Hibi and colleagues (1993) using a retroviral vector to introduce the v-Ha-*ras* gene into the non-tumorigenic FDC-P1 cell line. It was shown that infected FDC-P1 cells became not only tumorigenic but also showed increased monocytic differentiation *in vivo*. Differentiation and tumorigenicity *in vivo* was shown to correspond to an increase in activated *ras* gene expression. Although these cells displayed a more differentiated phenotype than the parental FDC-P1 cells, they did not undergo terminal differentiation, suggesting that the *ras* gene could be blocking differentiation at some later stage in the maturation pathway. The differentiation event which occurred was sufficient to further transform the cells and to confer tumorigenic capacity *in vivo*.

Alexander and colleagues (1992) demonstrated that karyotypic lesions were co-selected with differentiation *in vitro* and suggested that some of the genetic regions involved in transformation may exert a crucial regulatory role in growth and differentiation. However, it is also possible that not all changes are phenotypically detectable during the process of transformation, and that genetic events associated with growth and differentiation may take place without endowing the leukemic clone with an extrachromosomal 'product' which could serve as a qualitative or phenotypic marker to indicate their occurrence. The conclusion that can be drawn from these studies is that the dysregulation underlying the emergence of a leukemic clone might be quite subtle, and intimately linked with the target cell's program for differentiation (Greaves, 1986b).

1.5.3 Involvement of cellular genes: insertional mutagenesis associated with retroviral integration

Retroviruses exert mutagenic effects by integrating into the host genome, a natural step in the viral replication cycle. This process disrupts the host genome and may lead to misregulation of host genes by either activation or inactivation of a proto-oncogene (reviewed in Section 1.5.5). Three mechanisms by which retroviral insertion can activate proto-oncogenes have been identified. They are 3'LTR promotion, 5'LTR promotion and enhancer insertion. In 3'LTR promotion, the provirus is inserted at the 5' end of the proto-oncogene in the same transcriptional orientation as the gene. Transcription starts with the R and U5 regions of the 3' LTR and no other viral sequences are involved as they are largely deleted. In 5'LTR promotion, readthrough activation occurs and the oncogene transcripts usually contain viral leader sequences, since the 5'LTR is used to initiate transcription. Transcription therefore extends through the viral genome and through cellular sequences downstream of the insertion site. In enhancer activation, the orientation and positioning of the provirus is immaterial. The activated proto-oncogenes produce transcripts identical in size to their normal counterparts since they are initiated from natural or cryptic promoters (reviewed by Kung *et al.*, 1991).

Murine leukemia virus-induced leukemogenesis has been described as a series of genetic events whereby cell populations with distinct proviral integrations are continuously selected. The insertion of a provirus in or near specific cellular DNA domains has also been shown to be an important mechanism for virus-induced leukemogenesis in chickens and mice (Hayward *et al.*, 1981; Steffen, 1984; Cuypers *et al.*, 1984; O'Donnell *et al.*, 1985). Cellular genes brought under the control of powerful regulators of a newly integrated virus can become misregulated. Recombination between viral and cellular genes can result in the latter becoming oncogenic.

Proviral integration sites like *pim-1* (Cuypers *et al.*, 1984), *pim-2* (Breuer *et al.*, 1989), *Tpl-1* (Bear *et al.*, 1989) and *Tpl-2* (Patriotis *et al.*, 1993), *int-1* and *int-2* (Peters *et al.*, 1986) appeared to be involved in various stages of oncogenic progression. A high frequency of proviral integration in the *pim-1* region, for example, was observed to be associated with early T cell leukemogenesis (Cuypers *et al.*, 1984). Insertion near the *pim-2* locus was shown to be a late event in the development of MoMuLV-induced leukemia (Breuer *et al.*, 1989). Provirus integration in *Mlvi-4* by MoMuLV has been demonstrated to activate/affect expression of at least 3 genes, namely *c-myc*, *Mlvi-1* and *Mlvi-4*; the latter two by cis-acting mechanisms operating over long distances of the genomic DNA (Tsichlis *et al.*, 1990; Lazo, *et al.*, 1990).

T cell lymphomas induced by murine leukemia viruses often have several rearranged DNA domains due to independent proviral insertion events in the same tumour. These multiple integration events appear to be significant in oncogenesis. van Lohuizen (1989) showed that MuLV-infected transgenic mice (bearing the *pim-1* putative oncogene) developed T cell lymphomas much faster than similarly infected non-transgenic mice. In addition, all T cell lymphomas studied had either an activated *c-myc* or *n-myc* gene due to proviral insertion, indicating a strong cooperation between *pim-1* and *c-myc* in lymphomagenesis.

Similar synergism has also been demonstrated in rat thymic lymphomas induced by MoMuLV in which concomitant proviral integration in the *Mlvi-1* and *Mlvi-2* cellular DNA domains was observed (Tsichlis *et al.*, 1985). Breuer *et al.* (1989) found that insertion near *pim-2*,

which is a relatively late event in MoMuLV tumorigenesis, was also often preceded by proviral insertions in other common insertion sites such as *c-myc* within the same cell. Concerted activation of the two proto-oncogenes, *int-1* and *int-2* in carcinomas induced by mouse mammary tumour virus has also been found (Peters *et al.*, 1986). These studies indicate that synergistic events are important in oncogenic progression. Evidence such as this supports a multi-step model for leukemogenesis, in which each step requires activation of distinct cellular genes, with cooperation between oncogenes (Hunter, 1991).

Preleukemic and leukemic cell clones invariably undergo clonal selection *in vivo* as well as *in vitro*. Spontaneous viral re-integrations frequently occur, leading to acquisition of new proviruses (Seigel *et al.*, 1986, Cuypers *et al.*, 1986, Bear *et al.*, 1989). These observations have been questioned with respect to the significance of proviral insertions at the later stages of oncogenesis. Studies by Cuypers and colleagues (1986) on MoMuLV-induced T cell lymphomas have indicated that clonal selection is biased towards cells with further retroviral integration events. New integrations in the locus *Tpl-1* and *-2* (Tumour progression locus 1 & 2) at a late stage of oncogenesis were shown to confer growth advantage to affected cell clones over unaffected ones (Bear *et al.*, 1989; Patriotis *et al.*, 1993).

1.5.4 Retroviral recombination

Recombination between retroviruses is a frequent event observed after infection and integration into the host genome (Hu & Temin, 1990). This event has been reported to contribute to the oncogenic process. A case in point is the formation of tumorigenic MCF viruses in the thymus during the preleukemic phase in AKR mice (Hartley *et al.*, 1977). The presence of these recombinant viruses has been found to accelerate the onset of spontaneous leukemia (Cloyd *et al.*, 1980; Difronzo & Holland, 1993). The virological events leading to the generation of this leukemogenic agent were recently mapped by Stoye and colleagues (1991). This process was shown to be complex and precise, resulting in the generation of recombinant viruses with a defined structure. In the case of MoMuLV-induced leukemogenesis, it was also demonstrated that during outgrowth of

the tumour, clonal selection of cells carrying a certain recombinant proviral structure occurred. This structure was found to be conserved among many individual proviral recombinants and was considered to be a prerequisite for the onset of neoplasia (van der Putten, 1981).

MCF viruses generated by recombination have been shown to be leukemogenic in their own right. Homologous recombination between integrated proviruses can also result in chromosomal rearrangements. This has been reported for MoMuLV- and avian leukemia virus (AVL)-induced lymphomas (Lazo & Tscihlis, 1988; Ihle *et al.*, 1989; Nottenburg *et al.*, 1987). The resulting chromosomal aberrations were shown to contribute to tumour induction and progression.

1.6 Role of oncogenes in multistep carcinogenesis and leukemia

The association of oncogenes with proliferation and differentiation is important in the context of normal development and in oncogenesis. As both proliferation and differentiation are closely linked events in a cell's developmental program, it is very likely that any alteration in one process will affect the other.

A few studies have linked differentiation with proto-oncogene expression. Elevated expression of *c-myb*, for example, has been shown to prevent differentiation of Friend erythroleukemia cells after they were transfected with a *myb* expression vector (Clarke *et al.*, 1988). Studies on the *ras* and *src* oncogenes have also shown that they can suppress or block differentiation in a number of different cell types (reviewed by Boettiger, 1989). The specific mode of action is not clearly defined, but could relate to the growth factor receptor or cell signalling, at least for the *src* oncogene. Recently, elevated levels of *ras* expression have been associated with monocytic differentiation and tumorigenicity *in vivo* (Hibi *et al.*, 1993).

Evidence supporting the involvement of oncogenes in tumorigenesis has increased in recent years. For example, viral oncogene products have been found to share structural and functional similarities to growth factors

and their receptors. This was exemplified by the similarity between transforming protein $p28^{sis}$ of simian sarcoma virus and the platelet derived growth factor, PDGF (Waterfield *et al.*, 1983). Some oncogenes encode growth factors while others encode products which are signal transducers [H-*ras*, *c-mos*, *pim-1*, *v-src*, *v-abl*] or nucleoproteins [*c-myc*, *c-jun*, *c-myb*] (reviewed by Bishop, 1985; Butturini & Gale, 1990). The RB (retinoblastoma susceptibility gene) and the p53 gene represent a class of recessive oncogenes classified as tumour suppressor genes (Lee *et al.*, 1987; Donehower *et al.*, 1992). Both are known to be involved in cell cycling and signal transduction pathways, and there is increasing evidence for their involvement in many cancers.

Models have been constructed to study oncogene-driven tumour progression by introduction of exogenous oncogenes via either retroviral vectors or other forms of gene transfer into the cell. Their expression was then assayed during the course of transformation (Chisholm & Symonds, 1992; Bonham *et al.*, 1992; Hibi *et al.*, 1993). The data indicated that elevated expression of oncogenes was associated with the tumorigenic process. One of the mechanisms by which expression of an oncogene could contribute to oncogenesis was shown to be the induction of endogenous production of growth factors which could then stimulate the proliferation of the producer cell in an autocrine manner. Autocrine-driven cell proliferation could explain the transition from factor dependence to factor-independence in some transformed cell lines.

Insertional mutagenesis, point mutation, chromosomal translocation, deletion and amplification have been identified as some of the mechanisms by which cellular oncogenes can be misregulated during oncogenesis. In particular, translocations between or within chromosomes, deletion and amplification of chromosomal domains and chromosomal loss or gain are common genetic changes that have been identified in leukemias (Rowley, 1976). Translocations affecting proto-oncogenes are common in human leukemias and some of these could be attributed to location at or near breakpoints or fragile sites in the chromosome. A well studied example is the *c-myc* gene, which has been shown to be consistently translocated to various immunoglobulin genes in Burkitt's lymphoma (Leder *et al.*, 1983). Chromosomal abnormalities have also been linked to disease progression in

spontaneous AKR leukemias (Dofuku *et al.*, 1975; Wakonig & Stich, 1960) and in RadLV-induced lymphomas (Wiener *et al.*, 1978). In these cases, trisomy of chromosome 15 (and also 17 in the case of RadLV) was the identified genetic lesion. Direct evidence that karyotypic lesions were associated with differentiation and tumour progression of an immature thymic tumour cell was recently demonstrated *in vitro* by Alexander and colleagues (1992). Their studies suggested that the chromosomal regions involved have an important regulatory role in cell proliferation and/or differentiation.

1.7 Concluding remarks and objectives of this study

A feature common to many blood cell cancers is the uncoupling of normal proliferative and differentiative events, both of which are intimately linked in the cell's developmental program (Jacob, 1980). It has been shown that terminal differentiation in tumour cells such as teratocarcinomas, squamous cell carcinomas and myeloid leukemic cells can result in the reversal of malignancy and death of the cancer cell (Pierce & Wallace, 1971; Sachs, 1978; Jacob, 1980). In other cancers, further differentiative events have been associated with oncogenic progression (Section 1.5.2). Clearly the development of cancer is not the result of a single oncogenic event but rather a myriad of events which appear to proceed in concert in a step-wise fashion and which are likely to be influenced by the cellular microenvironment.

The discovery of oncogenes and tumour suppressor genes and their functional characterisation have been extremely valuable in expanding our understanding of the molecular mechanisms underlying the oncogenic process. Almost all cellular oncogenes identified so far are involved in cell development. The tumour suppressor gene, p53 is also involved in regulation of the normal cell cycle. It plays a critical role in arresting the mitotic cell-cycle in response to DNA damage arising from radiation or gene amplification. Mutation or loss of this gene results in loss of control, and cells continue in their mitotic cycle without DNA repair (Yin *et al.*, 1992; Livingstone *et al.*, 1992; Marx, 1994).

The objective of this study is to analyse oncogenic progression in T cell leukemogenesis using a retrovirally-induced murine lymphoid cell line, C1-V13D. This cell line was derived by *in vitro* infection of spleen cells with the thymotropic retrovirus, RadLV (O'Neill, 1992). These cells were shown to be capable of developing as a thymic lymphoma when injected intrathymically into mice. Using this cell line as a model, the nature of the target cell and genetic changes occurring during oncogenic progression were investigated. The role of the thymus, virus-host interactions and significance of further retroviral events in the process of oncogenesis were assessed. An attempt was made to isolate and characterise cellular genes in the vicinity of retroviral integration by constructing a genomic library from C1-V13D. This was initiated with the objective of uncovering novel cellular oncogenes which may play an important role in the transformation process.

CHAPTER 2

Investigating a model for oncogenic progression in T cell leukemia induced by retroviruses

2.1 Introduction

Despite numerous studies conducted to elucidate the mechanisms governing the oncogenic process, the factors which influence the proliferation of cells after infection with non-oncogene bearing retroviruses are still not fully understood. Most T cell leukemias and lymphomas induced by these slowly transforming viruses develop after a long latency period, and there is increasing evidence to indicate that the process of immortalisation and transformation of cells to neoplasia following infection involves sequential multi-step events (reviewed in Chapter 1).

It has been shown that RadLV can infect and immortalise lymphoid cells *in vitro* and that cell lines could be consistently derived within 8 weeks following infection (O'Neill, 1992). The use of established cell lines from lymphoid neoplasms as experimental 'tools' or models has provided many insights into the oncogenic process (reviewed in Section 1.3). This study was initiated to investigate the use of a RadLV-infected cell line, C1-V13D, as an *in vivo* model for studying oncogenic progression in T cell leukemogenesis. C1-V13D was established following *in vitro* exposure of mouse spleen cells to RadLV (O'Neill, 1992).

In this study, the capacity of C1-V13D to grow in different microenvironments was tested in selected strains of mice, including syngeneic DBA/2j and allogeneic mice such as CBA/H, which do not carry any endogenous RadLV or RadLV-like viruses, and C57BL/6J mice, which are susceptible to the RadLV virus. RadLV is known to induce T

cell lymphomas after a lengthy selection process in the thymus (Kaplan & Lieberman, 1976; Yefenof *et al.*, 1991; Kothler *et al.*, 1994). In an attempt to mimic this process, C1-V13D cells were passaged serially through the thymus. At each passage, C1-V13D cells were reisolated, subcloned and reinoculated into mice to test their ability to proliferate as a tumour *in vivo* following inoculation via various routes. This procedure has been utilised as it circumvents both the long latency period required by these viruses to induce leukemia *in vivo* and the added difficulty of establishing cell lines from RadLV-induced thymic tumours *in vitro* for future study. In this model, cell lines were tested for development of a more tumorigenic phenotype as an indication of oncogenic progression (Duhrsen & Metcalf, 1988; Yefenof *et al.*, 1991). This was manifested as increased ability to kill the host, irrespective of strain and route of inoculation, as well as ability to metastasise and grow subcutaneously.

2.2 Materials and methods

2.2.1 Origin and maintenance of cell lines

Details regarding the derivation of the C1-V13D cell line and isolation of V13, a cloned full length RadLV, have been described previously (O'Neill, 1992; Rassart *et al.*, 1986). C1-V13D originated from a DBA/2j mouse and was shown to have a unique hematopoietic phenotype resembling a precursor lymphoid cell (O'Neill, 1992). Sublines of C1-V13D were isolated from thymic tumours which developed following intrathymic injection of C1-V13D cells. They were cloned twice by limit dilution plating and maintained in supplemented Dulbecco's Modified Eagle Medium (sDMEM) containing 0.4% D-glucose, 0.006% Folic acid, 0.36% L-asparagine, 0.116% L-arginine hydrochloride, 0.015% L-glutamine, 0.2% sodium bicarbonate, 0.01M Hepes, 0.1M 2-mercapthoethanol, 0.2U/ml penicillin, 0.2mg/ml streptomycin and 10% foetal calf serum. Cells were passaged twice a week.

2.2.2. Mice

Mice of 4 to 8 weeks old were used in all experiments. They were bred under specific pathogen-free conditions in the John Curtin School of Medical Research, Canberra, Australia.

2.2.3 Electron microscopy

Cells (10^6) were prefixed for 2 hours using 2% glutaraldehyde (LADD, Australia) in 0.1M cacodylate buffer/pH 7.4, washed thrice with 0.1M cacodylate buffer and post-fixed for 90 minutes at 4°C in 1% osmium tetroxide. They were then stained *en bloc* in 2% uranyl acetate in water for 2 hours, washed thrice with water and dehydrated in increasing concentrations of alcohol. Infiltration with Spurr's resin (All-Tech Agar, Australia) mixed with alcohol (50 : 50) was carried out for 4 hours, followed by infiltration with 100% resin overnight. A further infiltration was carried out with fresh resin for another 4 hours, after which the tissue or cells were embedded in 100% fresh resin overnight at 70°C. Ultrathin sections (50nm-70nm) were stained with lead citrate (2.6% lead nitrate, 3.5% sodium citrate, 0.16M sodium hydroxide) for 10 minutes. Sections were viewed under an electron microscope (Philips, Model 301, Netherlands).

2.2.4 Fluorescent Activated Cell Sorter (FACS) analysis

The method for immunofluorescent staining of cells for FACS analysis was adapted from Coligan *et al.* (1992) with modifications. Briefly, 10^6 cells were incubated with specific antibody at 4°C for 30 minutes, washed thrice with sDMEM containing 0.1% sodium azide and then absorbed, as appropriate, with fluorescein isothiocyanate (FITC)-coupled sheep anti-rat Ig, goat anti-hamster Ig or sheep anti-mouse Ig (Serotec, Australia), for another 30 minutes at 4°C. Cells were washed thrice with medium, followed by one wash with phosphate buffered saline (8% NaCl, 1.25% $Na_2HPO_4.2H_2O$, 0.4% $NaH_2PO_4.H_2O$) and resuspended in fixative (1% paraformaldehyde in normal saline/pH 7.4). Stained cells were kept in the dark at 4°C and analysed within 48 hours.

Fluorescence was measured either on a FACS IV or a FACSCAN instrument (Becton Dickinson, Mountain View, CA) using standard filters. Data was analysed using either WEFCS software (Frank Battye, WEHI, Australia) or Winlist (Verity House Software, USA). Saturating concentrations of antibody were used to analyse determinant expression. Median channel number was used as a measure of determinant expression. The antibodies and reagents used for flow cytometry were: AT83, a rat IgM specific for Thy1.2 from R. Ceredig; GK1.5, a rat IgG_{2b} specific for CD4 from P. Marrack; 53-6.7, a rat anti-CD8 IgG_{2a} from J. Ledbetter; IM7.8.1, a rat IgG_{2b} specific for CD44 from I. Trowbridge; 145-2C11, a hamster antibody specific for CD3-ε from J. Bluestone; H57-597, a hamster antibody specific for TCR-αβ from R. Kubo; 34-1.2, a mouse IgG_{2a} specific for H-$2K^d$ from D. Sachs; RA3-6B2, a rat anti-B220 antibody from R. Coffman; 198D, a rat antiserum raised against C58NTD tumour which reacts with all MuLV proteins including gp70 from I. Weissman; M1/70, a rat antibody specific for Mac-1 from D. Hume; 30H-12, a rat IgG_{2b} specific for Thy1.2 purchased from Becton Dickinson (Mountain View, USA); F23.1, a mouse IgG_{2a} specific for TCR-$V\beta_{8.1,8.2,8.3}$ from M. Bevan. All monoclonal antibodies were prepared as hybridoma culture supernatants and concentrated where necessary by ammonium sulphate precipitation.

2.2.5 Preparation of a virus containing cell-free extract from C1-V13D

Virus was prepared as a cell-free extract by freezing C1-V13D cell cultures at -70°C and rapid thawing. This procedure was repeated thrice. The extract was centrifuged at 2000 x *g* for 30 minutes at 4°C to remove cell debris and the virus-containing cell-free supernatant was stored at -70°C in small aliquots.

2.2.6 Intrathymic injections

Four-week-old CBA/H, DBA/2j and C57BL/6J mice were sublethally irradiated (4Gy, cobalt source) and three hours later anaesthesized with a mixture of 2mg/ml Ketamine (Parnell Laboratories, Australia) and 10mg/ml Rompun (Bayer, Australia) in 0.2ml normal physiological saline. A small longitudinal midline incision was made in

the skin and through the sternum sufficient to expose the thymus. Cells, virus or medium (10µl) was injected into one lobe with a 36G needle affixed to a glass Hamilton syringe and the incision was sutured after expelling air from the thoracic cavity by gentle pressure on the upper abdomen. Mice usually recovered within a day and were given antibiotic water containing Polymyxin B sulphate (10^6U/L; Sigma, USA) and Neomycin sulphate (1.1g/L; Sigma, USA) for two weeks. Controls included unirradiated mice injected with virus and irradiated mice injected with medium. Animals were sacrificed and autopsied at various times after surgery or when symptoms of disease were apparent, i.e. ruffled fur, hunched posture, laboured breathing and weight loss. Thymic tumours were explanted and processed for FACS analysis or establishment of *in vitro* cultures.

2.2.7 Processing of explanted thymic tumours

Thymic tumours were removed under sterile conditions and rinsed in sDMEM. Single cell suspensions were prepared by gently pressing the tumour against a fine wire-meshed sieve. The cells were washed twice with sDMEM, resuspended in growth medium and cultured in 25cm^2 disposable tissue culture flasks (Corning Glass Works, USA). Autonomously growing cells were subcloned twice using limit dilution plating.

For histological analysis, thymic tumours were fixed in formalin/saline immediately upon removal, embedded in paraffin wax and sectioned. The sections were stained using Haematoxylin and Eosin (H&E).

2.2.8 Southern analysis

2.2.8A Preparation of genomic DNA

DNA was prepared according to Blin & Stafford (1976). Cells (10^9) were washed thrice with phosphate buffered saline, resuspended in lysis buffer (0.2M Tris, 0.2M EDTA, 1% sodium N-lauroyl sarcosine) containing 0.1% freshly prepared Proteinase K and incubated at 50°C for

6-8 hours. Five mls of TE buffer (0.01M Tris/pH 7.4, 0.001M EDTA/pH 8.0) was then added and the DNA was kept at 4°C. Processing involved the extraction of DNA twice with an equal volume of TE-saturated phenol:chloroform:isoamyl alcohol (24:1:1), followed by extraction with chloroform. Five mls of 10M ammonium acetate was added and the DNA was precipitated by adding an equal volume of isopropanol. The DNA pellet was washed twice with 95% ethanol, dried in a rotary concentrator (Speed Vac, model #100H, Savant Instruments, USA) for approximately 15 minutes. The pellet was dissolved in 500µl TE buffer at 4°C overnight.

2.2.8B Endonuclease restriction and agarose gel electrophoresis

DNA (10µg) was restricted using enzyme concentrations of ≤ 4U/µg DNA for 4 to 16 hours under optimal conditions (as recommended by the supplier). The DNA was resolved by electrophoresis in agarose (Type II, Sigma, USA). Prior to loading, DNA samples were mixed with sample loading buffer (0.01% Bromophenol Blue, 38% sucrose, 0.067M EDTA/pH 8.0), heated for 10 minutes at 65°C and chilled briefly on ice. The gels were run at a constant voltage of between 25 and 30 volts for 18-24 hours using TAE (0.04M Tris, 2.3% glacial acetic acid, 0.02M EDTA/pH 8.0) as electrophoresis buffer.

2.2.8C Transfer of DNA to membranes

Fractionated DNA was transferred by capillary action from agarose gels to Hybond-N^+ membranes (Amersham, Australia) using 0.4M NaOH (Reed & Mann, 1985). Transfer time varied between 6 to 16 hours. Gels were soaked for 10 minutes in 0.25M HCl and rinsed briefly in ddH_2O before transfer. After DNA transfer, membranes were rinsed briefly in 2XSSC (0.3M NaCl, 0.03M tri-sodium citrate/pH 7.0), vacuum baked for 2 hours at 80°C and stored dry between sheets of 3mm Whatman paper in sealed bags at -20°C.

2.2.8D Synthesis of ^{32}P-labelled probes

DNA probes were made using purified inserts as templates by the random priming method (Feinberg & Vogelstein, 1983) using

[α^{32}P]-dCTP (3000Ci/mmol specific activity; Amersham, Australia). For labelling purposes, up to 100µCi of the radiolabel was used per 100ng of template DNA. The insert was boiled for 5 minutes, chilled immediately on ice followed by the addition of [α^{32}P]-dCTP and 12.4µl of LSB buffer, containing 11.4µl LS mix and 1µl BSA (10mg/ml). LS mix was prepared by combining 25 parts 1M Hepes/pH 6.6, 25 parts DTM solution (100µM each of dATP, dGTP, dTTP [Pharmacia, USA], 0.25M Tris/pH 8.0, 0.05M 2-Mercaptoethanol, 0.025M $MgCl_2$) and 7 parts OL solution [1mM Tris/pH 7.5, 1mM EDTA, 90 OD/ml oligo-deoxyribonucleotides (Hexamers-pd$(N)_6$; Pharmacia, USA)]. Synthesis was carried out at room temperature for 6-18 hours using 5 Units of DNA Polymerase I, Klenow fragment (Pharmacia, USA). Unincorporated label and nucleotides were separated from the labelled product by passage through a Sephadex G-50 column using STE buffer (0.01M Tris/pH 7.4, 0.59% NaCl, 0.001M EDTA/pH 8.0) as eluant. A specific activity of $1x10^7$ to $5x10^7$ cpm/µg of template DNA was routinely achieved.

2.2.8E Detection of specific nucleic acid sequences

Both pre-hybridisation and hybridisation reactions were carried out in glass roller bottles at 42°C in a hybridisation oven (Hybaid Oven Model "Maxi-14"; Integrated Sciences, Australia) for 16-18 hours. Prior to hybridisation with labelled probes, blots were pre-wetted with 2XSSC and then equilibrated with pre-hybridisation cocktail comprising 50% Formamide, 3XSSC, 1% SDS, 10X Denhardt solution [0.01% Ficoll 400 (Pharmacia, USA), 0.01% Polyvinyl Pyrrolidone (BDH Laboratory Reagents, Australia), 0.01% BSA] and 0.1mg/ml salmon sperm DNA (Type III; Sigma, USA). The cocktail was removed and replaced with hybridisation cocktail (50% Formamide, 3XSSC, 1.5% SDS, 1X Denhardt solution, 9.9% dextran sulphate [Sigma, USA]) containing heat denatured ^{32}P-labelled probe (boiled for 5 minutes) at $5x10^6$ to 10^7 cpm/ml. Blots were washed under conditions of high stringency, ie. twice with 2XSSC/0.1% SDS for 10 minutes at room temperature and then twice with 0.2XSSC/0.5%SDS for 15 to 20 minutes at 65°C. Specific signals were visualized by exposure for an appropriate time to X-ray film (RP X-Omat; Kodak, Australia) at -70°C with intensifying screens (Dupont, USA). Blots were stripped twice with boiling buffer (0.01M Tris, 0.1mM EDTA, 0.023%

SDS/pH 8.0) after use and stored in sealed bags at -20°C. For reprobing, blots were returned to room temperature and soaked briefly in 2XSSC before pre-hybridization. The Vα3 probe used in this study was derived from the C6VL/1 lymphoma cell line. It is a 468 bp fragment derived from the 5' end of the Vα3 cDNA to the *Apa*LI site. It is a new member of the Vα3 family and was cloned by Dr. Chris Jolly in this laboratory into a pGEM^R plasmid vector (unpublished results).

2.2.9 Statistical methods

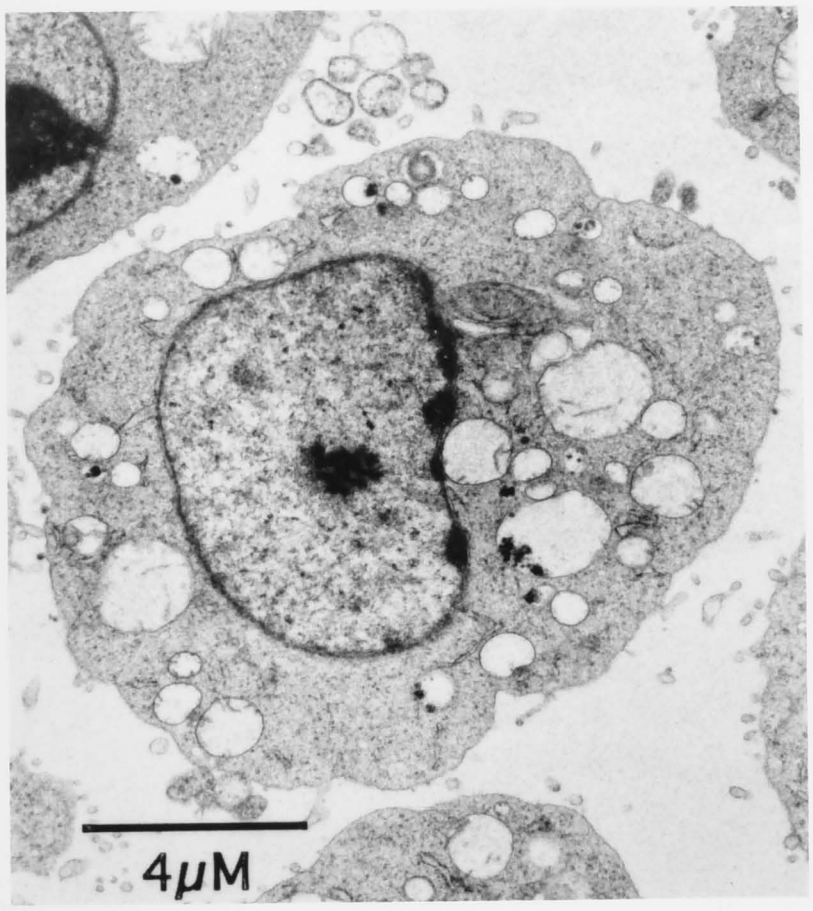
The Wilcoxon Rank Sum Test (1-sided) has been used to determine statistical significance. This analysis uses ordering of the actual times to death and therefore takes into account all information on survival, ie. time and mortality. The analysis was performed by Dr. Terence J. O'Neill from the Statistical Science Program, Centre for Mathematics and its Applications, Australian National University, Canberra.

2.3 Results

2.3.1 Characteristics of C1-V13D by electron microscopy and FACS analysis

C1-V13D cells are factor-independent, grow autonomously in culture and attach loosely to the surface of the flask. Unlike plasma and epithelial cells (Wheater *et al.*, 1987), C1-V13D cells have essentially little or no endoplasmic reticulum. Instead the cytoplasm is filled with numerous free ribosomes and enlarged mitochondria. Electron micrographs of C1-V13D in comparison to a normal thymocyte are shown in Fig. 1. The cells are approximately 2-3X larger than normal thymocytes with chromatin arranged in a thin perinuclear fashion in the large euchromatic nucleus. There is little evidence of virus being produced although C1-V13D carries full length integrated ecotropic RadLV-like viruses (Fig. 3, Chapter 4).

a)



b)

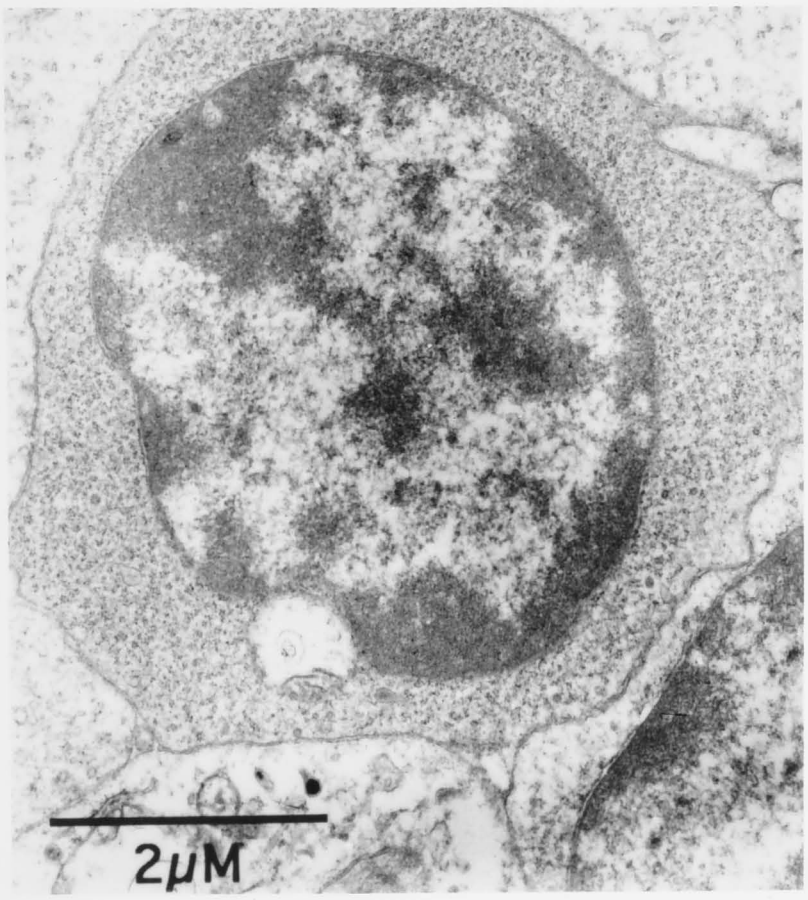


Fig. 1 Transmisson electron micrographs of (a) C1-V13D and (b) a normal thymocyte. C1-V13D cells are 2-3X larger than normal thymocytes and the cytoplasm is filled with numerous ribosomes and mitochondria. Chromatin is arranged in a tightly packed fashion along the nuclear membrane.

The phenotype of C1-V13D, as determined by a panel of monoclonal antibodies and antisera against lineage specific determinants, is summarised in Table 1. The phenotype is confirmed in this study to be Thy-1.2$^-$ CD4$^-$/8$^-$ CD3-ε$^-$ TCR-αβ$^-$ Mac-1$^-$, with high expression of CD44, B220, NK1.1, T200 and the H-2K^d MHC class I molecule, as reported by O'Neill (1992). C1-V13D cells also express high levels of the MuLV envelope gp70 protein. In contrast, thymocytes do not express B220, and CD44 is expressed by only a small percentage of thymocytes in both DBA/2j and CBA/H mice. All DBA/2j thymocytes, but not CBA/H thymocytes, strongly bound the 34-1.2 antibody specific for H-2K^d (Table 1).

2.3.2 C1-V13D cell-free extract contains a leukemogenic agent(s)

The cell-free extract of C1-V13D was found to be highly leukemogenic when inoculated intraperitoneally into newborn C57BL/Ka mice. Approximately 85% of inoculated animals developed thymic tumours with an average latency period of 12.2 months (Table 2). This suggested the presence of a thymotropic agent. In contrast, this same extract was only weakly leukemogenic when inoculated intrathymically into irradiated adult CBA/H mice. Only 2 out of 15 mice succumbed to disease and death at 11 and 12 months post-infection. In both cases, enlarged livers and spleens were observed in the absence of any thymic tumours. Adult CBA/H mice were relatively resistant to infection by RadLV. In comparison, the transfer of C1-V13D cells intrathymically gave rise to thymic tumours within 2 to 3 weeks. Survival time was therefore significantly greater for mice inoculated with the cell-free extract from C1-V13D than age-matched mice inoculated with C1-V13D cells (Tables 2 & 3; $p=7.77\times10^{-6}$). It was therefore very unlikely that these thymic tumours were of host origin, arising secondarily as a result of virus transferred from C1-V13D.

2.3.3 Growth capacity of C1-V13D in vivo

C1-V13D (5×10^{5}) cells were inoculated into various strains of mice and via various routes. In DBA/2j mice, enlargement of the

TABLE 1 Phenotype of C1-V13D compared with thymocytes from CBA/H and DBA/2j mice

	Antibody binding (Median Channel Number by FACS analysis)												
Controls	198D MuLV	AT83 Thy1.2	GK1.5 CD4	53-6.7 CD8	IM7.8.1 CD44	145-2C11 CD3-ε	H57-597 TCR-αβ	34-1.2 MHC Class I	RA3-6B2 B220	PK136 NK1.1	M1/70 Mac-1	30G-12 T200	F23.1 $TCR_{V\beta 8.1, 8.2, 8.3}$
C1-V13D	52	-	-	-	55	-	-	85	40	41	-	53	-
CBA/H thymus	5	97	10/66	21/97	-/22	21/82	18/74	-	-	-	ND	-/117	-/46
DBA/2j thymus	5	90	36/92	63/131	-/36	42/119	15/91	63	-	20/66	ND*	-/122	-/51

Numbers represent specific fluorescence values after subtracting background binding. To determine background binding, sDMEM was used in place of specific antibody. When 2 values are shown, these represent the two major thymic subsets with different levels of determinant expression. Channel shifts of $\leq$ 4 are considered as background staining and are presented as "-".

*Bone marrow, used as a positive control, resulted in a median channel shift of 123.

ND: Not done

TABLE 2 Leukemogenicity of virus present in a cell-free extract of C1-V13D

Mouse strain	Age	Inoculation route	Tumour incidence	Time to death (months)
C57BL/Ka	newborn	ip	6/7[a]*	9,10,11,12,13,18
CBA/H	adult	it	2/15 [a]**	11,12

A cell-free extract (10µl) of C1-V13D cells was tested for the presence of leukemogenic virus(es).

* Only thymic tumours were observed.

** Enlarged livers and spleens were observed.

a Statistically significant ($p \leq 0.0193$)

spleen, liver and sometimes the lymph nodes occurred following intraperitoneal (ip), intravenous (iv) or subcutaneous (sc) inoculation. Irradiation was found to significantly shorten survival time of syngeneic DBA/2j mice inoculated with C1-V13D by each of these routes. C1-V13D cells were unable to grow subcutaneously in unirradiated DBA/2j mice (Table 3).

C1-V13D was not tumorigenic in two allogeneic mouse strains tested, namely C57BL/6J and CBA/H, when inoculated via the ip, sc or iv routes. In contrast, C1-V13D cells had the specific capacity to grow as a thymic tumour when inoculated directly into the thymus of irradiated allogeneic CBA/H (Table 3) or C57BL/6J x CBA/H(F1) mice (data not shown). Irradiated 4 to 5 week-old mice died from massive thymic tumours occurring within 2-3 weeks post-inoculation (Fig. 2) and with an incidence of 80% in CBA/H mice. The incidence was significantly lower in C57BL/6J x CBA/H(F1) mice ($p \leq 0.00549$; data not shown). This could reflect the involvement of *Hh* (hybrid histocompatibility) genes (Cudkowicz *et al.*, 1983). No other organs or lymph nodes appeared to be involved. The lethal dose of cells was determined at $5x10^4$-10^5 cells, with the incidence of tumour formation decreasing with age (data not shown). No deaths were observed in unirradiated mice whichever the route of inoculation (Table 3).

2.3.4 Thymic tumours were identified as lymphomas by histology

Thymic tumours arising from intrathymic inocultion of C1-V13D lacked the clear cortex/medulla delineation of normal thymus. Foci of necrosis were also observed and there was a noticeable absence of normal lymphocytes. The cells that made up the tumour were identified as lymphoblasts, indicating that the tumour was a lymphoma. A section of a thymic tumour, which developed following inoculation of C1-V13D cells, is compared with that of a normal CBA/H thymus in Fig. 3 (H&E staining).

TABLE 3 Tumorigenicity of C1-V13D in mice

Strain	Mortality and time to death (days)							
	intraperitoneal		intravenous		subcutaneous		intrathymic	
	I	UI	I	UI	I	UI	I	UI
DBA/2j	3/3 (14,15[x2])[a]	2/3 (21,25)[a]	3/3 (13,14[x2])[b]	2/3 (21,49)[b]	3/3 (22,25,28)[c]	0/3[c]	2/2 (13,15)	2/2 (14,33)
CBA/H	0/3	0/3	0/3	0/3	0/3	0/3	12/15 (13,14[x7], 16[x2],21[x2])	0/5
C57BL/6J	0/3	0/3	0/3	0/3	0/3	0/3	NT	NT

Deaths were scored for up to 5 months following inoculation of $5x10^5$ C1-V13D cells. Four week-old mice were used.

NT: Not tested

I: Irradiated, whole body (4 Gy)

UI: Unirradiated

a,b,c Statistically significant at the 5% level by the Wilcoxon Rank Sum Test (1-sided)

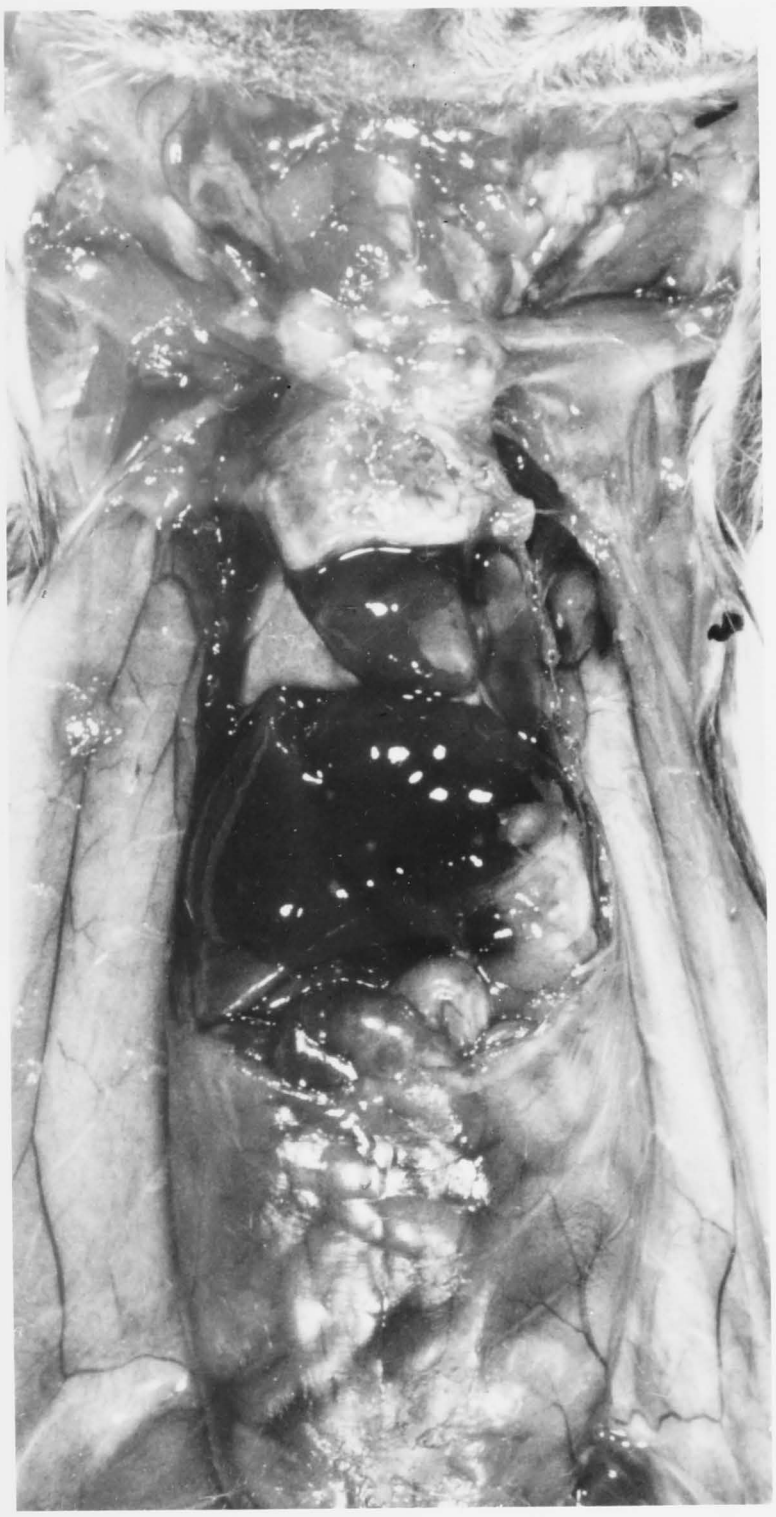
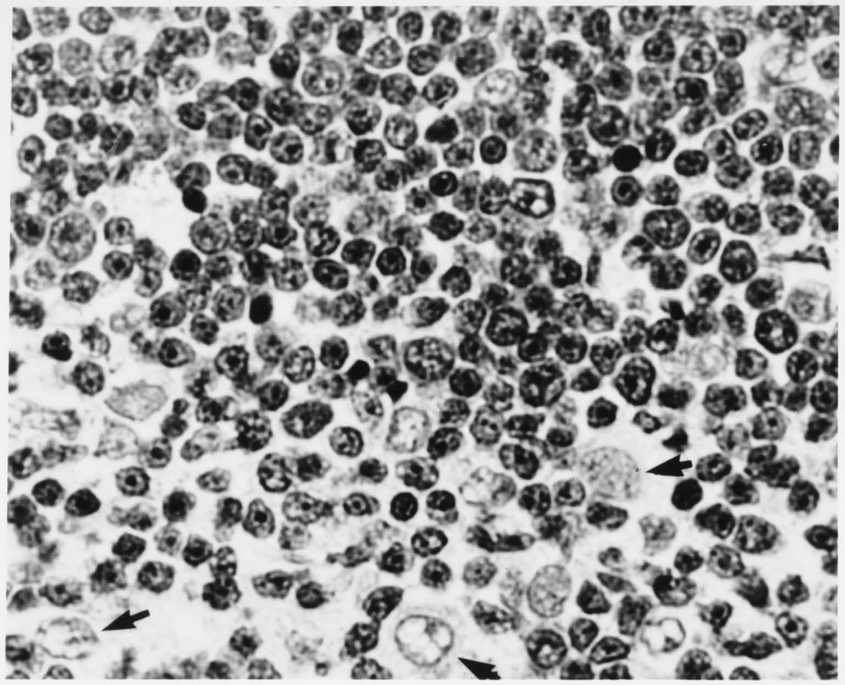


Fig. 2 Typical thymic tumour present in a CBA/H mouse inoculated intrathymically with $5x10^5$ C1-V13D cells. No other organs appeared to be involved.

a)



b)

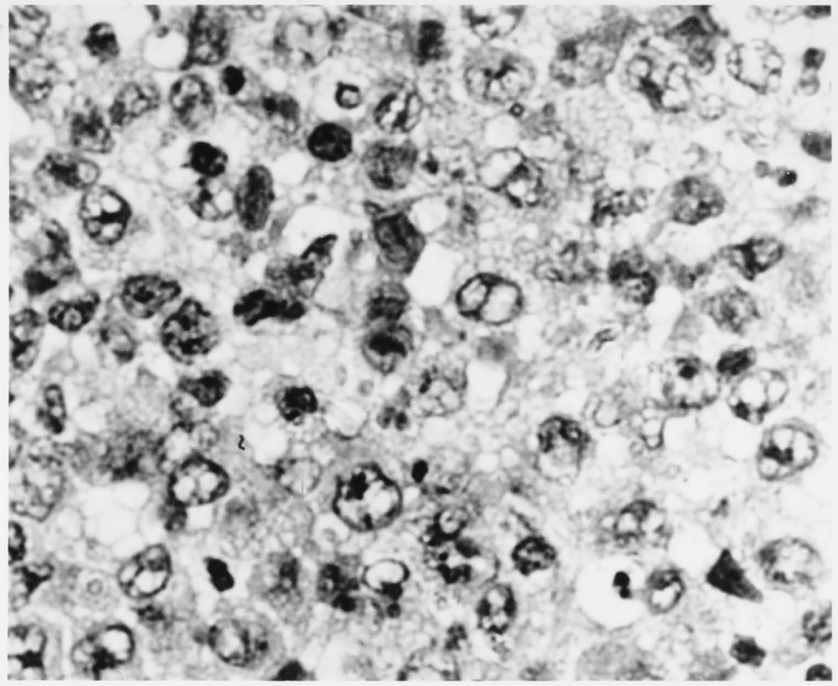


Fig. 3 Haematoxylin & Eosin stained sections of a normal thymus and a thymic lymphoma which developed following inoculation of C1-V13D cells.
a) The corticomedullary junction of a normal thymus showing small numbers of lymphoblasts (arrowed) scattered amongst the smaller lymphocytes in the cortical region.
b) A typical thymic lymphoma showing the large lymphoblasts which make up the bulk of the tumour. Little or no normal lymphocytes were observed.
(Magnification: 650X)

2.3.5 *Cell lines derived from explanted thymic tumours phenotypically resembled C1-V13D*

Autonomously replicating cells could be isolated from thymic tumours within 2 days of *in vitro* culture. These were subcloned twice prior to cell surface marker analysis by flow cytometry. All sublines isolated this way were found to have the same cell surface phenotype as C1-V13D. Data obtained from 4 primary clones, each isolated from a different thymic lymphoma is shown in Table 4. All clones remained Thy-1.2$^-$ CD4$^-$/8$^-$ CD3-ε$^-$ TCRαβ$^-$ and expressed high levels of B220, CD44, RadLV gp70 envelope protein and the Class I H-2K^d epitope, indicating their clonality and donor origin.

2.3.6 *Increased tumorigenic potential of C1-V13D cells following thymic residence*

Clones isolated from thymic lymphomas following one intrathymic passage into CBA/H mice and which phenotypically resembled the parental C1-V13D were found to be more tumorigenic when injected back into irradiated CBA/H mice (Table 5). Mortality ranged from 33% to 100% for different clones. Mice injected intraperitoneally died with liver and gut lesions (Fig. 4a), and phenotypically similar cells could be re-isolated from the ascitic fluid of these animals (data not shown). Enlarged spleen, lymph nodes and liver, with tumour cells infiltrating into the liver were observed in all mice inoculated intravenously (Fig. 4b). The thymus was regressed in both cases. Subcutaneous inoculations did not produce any local or generalised tumours.

The tumorigenic potential of C1-V13D was observed to increase further with serial passage through the thymus. This was demonstrated using ET4-D, a subline cloned from the T4 thymic lymphoma (Table 4). Serial passage involved the re-isolation of a single clone after each passage, phenotypic characterisation by FACS analysis, and injection back into the thymus. The second and third passage clones have been named ET4-D(T1-E) and ET4-D(T1-E)M7A, respectively. The origin of these cloned lines was confirmed as C1-V13D by their distinct

TABLE 4 Phenotype of autonomously replicating cell lines islolated from four different primary thymic tumours

		Antibody binding (Median Channel Number by FACS analysis)								
Tumour (clone)	Host strain	198D MuLV	AT83 Thy1.2	GK1.5 CD4	53-6.7 CD8	IM7.8.1 CD44	145-2C11 CD3-ε	H57-597 TCR-αβ	34-1.2 MHC Class I	RA3-6B2 B220
T1(ET1-A)	CBA/H	39	-	-	-	43	-	-	ND	ND
T2(ET2-D)	CBA/H	45	-	-	-	43	-	-	63	45
T3(ET3-B)	CBA/H	49	-	-	-	49	-	-	85	50
T4(ET4-D)	CBA/H	49	-	-	-	49	-	-	83	43
Control thymocytes	CBA/H	-	95	15/73	16/95	13	21/92	18/71	-	-

Numbers represent specific fluorescence values after subtracting background binding, as described in Table 1.

Channel shifts of ≤ 4 have been considered as background staining and are presented as "-".

ND: not determined.

TABLE 5 Increased tumorigenicity of C1-V13D clones following intrathymic replication

Tumour	clones*	% mortality (time to death, range in weeks) intraperitoneal	intravenous	subcutaneous
T1	ET1-A	100 (2-3)	33 (3)	0
T1	ET1-C	33 (8)	33 (7)	0
T1	ET1-D	100 (4-6)	0	0
T2	ET2-D	66 (3)	33 (2)	0
T3	ET3-B	66 (2-3)	66 (2)	0
T4	ET4-D	33 (2)	100 (2)	0
-	C1-V13D	0	0	0

T1, T2, T3 and T4 are different thymic tumours arising from the first intrathymic passage of C1-V13D cells into irradiated CBA/H mice.

* Clones were isolated after *in vitro* culture and limit dilution cloning of thymic tumours. To test for tumorigenicity, $5x10^5$ cells of each cloned line were inoculated into 4-week old irradiated (4Gy, cobalt source) CBA/H mice.

Fig. 4 C1-V13D cells acquired increased tumorigenic potential following intrathymic growth. Typical lesions resulting from inoculation of a clone derived from C1-V13D into 4Gy irradiated allogeneic CBA/H mice are shown.

a) Intraperitoneal inoculation
Infiltration of tumour cells into the gut and liver was observed as white patchy growths. The thymus was extremely regressed.

b) Intravenous inoculation
Infiltration of C1-V13D cells into the enlarged liver was evident as white nodules. The submaxillary and inguinal lymph nodes were also enlarged. The thymus was extremely regressed.

RFLP (Restriction Fragment Length Polymorphism) pattern at the TCRα locus, which is different in the CBA/H host strain (Fig. 5). The passage of these cells through the thymus gave rise to cells of increasing tumorigenic potential. The primary clone, ET4-D, was significantly more tumorigenic than C1-V13D by the intravenous route and the second passage clone, ET4-D(T1-E), was significantly more tumorigenic than C1-V13D by the intraperitoneal route (Table 6). By the third passage, several subcutaneous tumours were observed at or near the site of inoculation, indicating proliferation of C1-V13D cells and some degree of metastasis. Subcutaneous growths were not observed with the primary (ET4-D) or secondary clones (ET4-D[T1-E]) throughout this period of study.

2.4 Discussion

The capacity of C1-V13D to grow only in the thymus of allogeneic CBA/H mice suggests an important role for the thymus in the oncogenic progression of a RadLV-infected cell line. By using the allogeneic CBA/H model, C1-V13D cells could be distinguished clearly from host thymocytes by cell surface marker expression and also by the different RFLP pattern at the TCR-α locus, using Southern analysis. This property of thymic replication is particularly important since C1-V13D resembles a precursor lymphoid cell type.

Electron microscopy showed that C1-V13D resembles an immature lymphoblastic cell. This immature phenotype was further confirmed by FACS analysis using a panel of antibodies against cell surface markers expressed by hematopoietic cells. This result is consistent with earlier findings that immature cells are more susceptible to retrovirus infection and are targets for infection and initial transformation by many leukemogenic viruses (reviewed in Section 1.4). High cell surface expression of CD44 and NK1.1 are not uncommon in tumour cells. NK1.1 is highly expressed on cells infected with LP-BM5, a murine leukemia virus which induces immunosuppression (Markino *et al.*, 1993). CD44 is strongly transcribed in many tumours as a splice variant (Herrlich *et al.*, 1993) and also in >95% of childhood leukemic cells (Kreindler *et al.*,

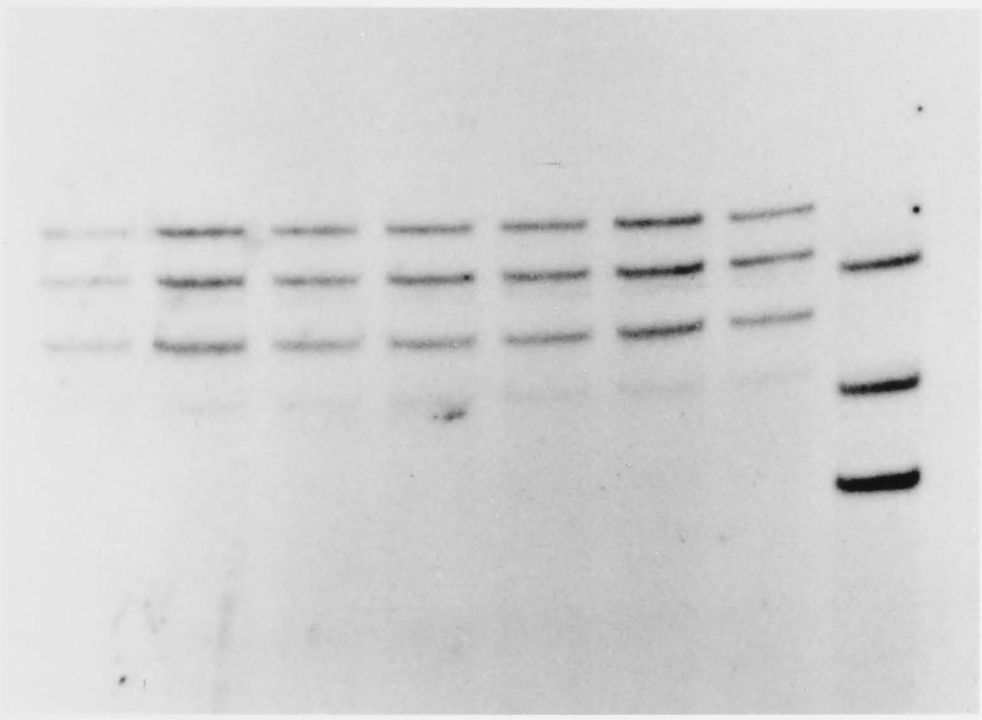


Fig. 5 Southern analysis to compare the RFLP pattern at the TCRα locus in CBA/H thymus (Thy), C1-V13D and subclones isolated upon 1st (ET4-C, ET4-D), 2nd (ET4-D[T1-C], ET4-D[T1-E]) and 3rd (ET4-D[T1-E]M7A, ET4-D[T1-E]M7C) passage intrathymically. DNA was digested to completion with *Eco*RI. The probe used was a cDNA specific for the Vα3 gene. The migration of *Hind*III - digested λ DNA (kb) is indicated.

TABLE 6 Increasing tumorigenic potential of C1-V13D following serial intrathymic passage into irradiated CBA/H mice

Clone	Thymic Passage Number	Incidence of thymic tumour formation (time to death, range in days) intravenous	intraperitoneal	subcutaneous
ET4-D	1	3/3(16[x3])[a]	1/3(16)	0/3
ET4-D(T1-E)	2	3/3 (10[x2],14)	4/4 (17[x3],18)[b]	0/3[c]
ET4-D(T1-E)M7A	3	3/3 (10,16[x2])	3/3 (15,16[x2])	0/3[c]*
C1-V13D	-	0/3(>4.5m)[a]	0/3(>4.5m)[b]	0/3

CBA/H mice were irradiated (4Gy, Cobalt source) and inoculated with $5x10^5$ C1-V13D cells.

* Tumours were evident subcutaneously at 10-14 days post-inoculation but regressed within 4 weeks.

a,b Statistically significant at the 5% level: day to death

c time of tumour detection.

m months

1990). The CD44 antigen is also expressed on early fetal thymocytes and thymus-homing progenitor cells (Lesley *et al.*, 1985a & b). C1-V13D cells co-express high levels of B220 and T200, lymphoid antigens which are specific markers for cells of the B (Coffman & Weissman, 1981) and T lineages (Thomas, 1989), respectively. The co-expression of related but different lineage antigens, which at first sight appears erroneous could be explained by lineage promiscuity (reviewed in Section 1.4), an intrinsic property of immature hematopoietic cells. This evidence indirectly lends support to the immature status of C1-V13D.

Irradiation has been shown to temporarily suppress the host's immune response (Miller *et al.*, 1988), restoring the adult immunologic responsiveness to near neonatal level (Lieberman & Kaplan, 1976b). The transplantability of C1-V13D cells into sublethally irradiated syngeneic DBA/2j mice by various routes, but not with the same efficiency via subcutaneous route into unirradiated DBA/2j mice, suggests that C1-V13D cells are not fully transformed. Using non-leukemogenic irradiation doses, the non-tumorigenic, factor-dependent FDC-P1 cell line has also been demonstrated to grow as a tumour in irradiated syngeneic mice (Duhrsen & Metcalf, 1988). The capacity of C1-V13D to grow as a tumour was shown to be dependent on two factors: (a) an immune-suppressed host induced by irradiation and (b) the route of inoculation. C1-V13D was unable to grow in irradiated or unirradiated allogeneic CBA/H and C57BL/6J mice following inoculation via the sc, ip or iv routes. MHC differences and an immune response could account for this restriction. However, in irradiated CBA/H mice, C1-V13D replicated successfully when inoculated intrathymically. This observation supports the hypothesis that thymus may be an immunologically privileged site, which can function as a safe haven for emergence, survival and proliferation of leukemic cells. In this model, irradiation would have depleted the thymus of mature T cells (Huiskamp *et al.*, 1985), further enhancing the growth of C1-V13D cells in the absence of any cell-mediated immune response. The irradiation regime employed in this study (4 Gy) does not induce spontaneous leukemia (Kaplan & Brown, 1952), nor was the physical manipulation a triggering mechanism for leukemogenesis because control mice injected with medium in place of cells did not develop tumours (data not shown). Since cells which

replicate in the thymic microenvironment developed increased oncogenic potential, this would suggest an important role for the thymus in oncogenic progression. As shown here, this can be best demonstrated in the allogeneic model.

Since RadLV is a thymotropic virus and can replicate efficiently and productively in the thymus, it was important to confirm that cells reisolated are not host cells which have become secondarily infected by RadLV. Cells reisolated from thymic tumours were verified as C1-V13D progeny by several lines of experimental evidence. Firstly, cell-free extracts from C1-V13D were shown to produce a leukemogenic virus(es) which was tumorigenic in newborn C57BL/Ka mice. This same extract was only weakly leukemogenic in irradiated adult CBA/H mice, giving tumours only after a long latency period of approximately 1 year, and with a mere 13% incidence of leukemia and little thymic involvement. The results are consistent with reported findings that RadLV requires a long incubation period to induce lymphomas after infection and that adult CBA/H mice are relatively resistant to RadLV [(<25% develop leukemia following infection with virus (Kaplan, 1967)]. In contrast, the inoculation of C1-V13D cells into the thymus at a dose of $5x10^4$ to $5x10^5$ cells resulted in localised tumour formation within 2-3 weeks and rapidly growing cells could be established within 24 hours of *in vitro* culture. The coincident proliferation of other RadLV infected cells in the thymus is not disputed. However, the establishment of such cells in culture would require a much longer period of time.

Conclusive evidence that the clones reisolated from thymic tumours were the progeny of C1-V13D came from FACS and Southern analyses. All clones expressed the same markers as C1-V13D upon subcloning and also demonstrated an RFLP pattern similar to C1-V13D at the TCR-α locus, which is distinct from the allogeneic CBA/H host. *However, C1-V13D progeny clones exhibited increasing tumorigenic potential with each passage through the thymus and progressed sufficiently to grow subcutaneously after the third passage, suggesting at each step the occurrence of distinct oncogenic changes.*

This model lends itself to analyses aimed at elucidating events in retrovirus-induced oncogenesis, using cells isolated at various stages of transformation. The results presented here already provided evidence consistent with the involvement of the thymus in T leukemogenesis induced by T cell tropic retroviruses. They also indicate how the thymus can act as an appropriate microenvironment for the survival and proliferation of leukemic cells in an immune suppressed allogeneic host, presumably via adaptation and progression to a more tumourigenic phenotype. Further experiments, aimed at analysing changes in C1-V13D during oncogenic progression *in vivo*, as well as the genes associated with the transformed phenotype, are presented in the following chapters.

SUMMARY

The objective of this study was to investigate the use of a RadLV-infected cell line, C1-V13D, as a model for studying oncogenic progression in T cell leukemogenesis. *In vivo* studies indicated that C1-V13D is partially transformed but can undergo further progressive transformation at each serial passage through the thymus, thus confirming a role for this organ in oncogenesis. The isolation of progressively tumorigenic C1-V13D cells allows for a step-by-step dissection of events contributing to multi-step carcinogenesis, and on this basis, C1-V13D could serve as a useful model for the study of T cell leukemogenesis.

CHAPTER 3

Oncogenic progression: A differentiation - linked event?

3.1 Introduction

Cells susceptible to productive infection by RadLV are present amongst the most immature cell subset in the thymus (Boniver *et al.*, 1989). However, cell lines established from RadLV-induced thymic tumours often exhibit characteristics of more mature T cell phenotypes (Lieberman *et al.*, 1979; Yefenof *et al.*, 1991). This is consistent with observations that leukemic cells shift towards a more mature phenotype during disease progression (O'Donnell *et al.*, 1984; Davis *et al.*, 1986a; Jensen *et al.*, 1992). Leukemic cell lines have also been reported to exhibit unstable phenotypes and continue to differentiate further (Koeffler & Golde, 1978; Lazo *et al.*, 1990). In the case of chronic myelogenous leukemia (CML), it was observed that expansion of the leukemic cell population takes place in the intermediate and more mature cell compartments rather than in the stem cell or early progenitor compartments, where the disease is known to originate (Clarkson & Strife, 1993). These observations are consistent with the hypothesis that cell differentiation is intrinsic to multistep leukemogenesis (reviewed in Section 1.5.2).

The mechanisms underlying differentiation-linked oncogenesis are not clear. Some genes that regulate growth, differentiation and cell death have been mapped to unstable 'breakpoint' regions of chromosomes which are prone to mutation, recombination, translocation or deletion (Leder *et al.*, 1983; Yunis, 1983; Hatano *et al.*, 1991; Reed, 1994). Some of these alterations have been shown to occur spontaneously and with striking association between particular chromosome alterations and certain cell types (Rowley, 1990). For example translocations involving the TCR-α locus have been found to be associated with T cell neoplasms (Croce *et al.*, 1985).

Burkitt's lymphoma in humans is another example. In this case, the Epstein Barr virus is thought to stimulate proliferation of B cells which are targets for additional neoplastic events involving translocation of the *c-myc* proto-oncogene next to the fragile rearranging immunoglobulin gene locus (Magrath, 1990). The mechanism for differentiation-linked oncogenic progression could therefore involve alteration of genes important in cell differentiation. These genes would be unique to the developmental program of the particular cell involved.

Data presented in the previous chapter indicated a role for the thymus in oncogenic progression since C1-V13D cells were shown to be progressively more tumorigenic after each successive intrathymic passage. This was reflected by a change towards a more tumorigenic phenotype when the cells were allowed to replicate under the selective pressure of the thymic microenvironment. This study is aimed at investigating differentiation-linked oncogenic progression in C1-V13D. To this end, the capacity of C1-V13D cells to undergo further differentiation and to express markers of T cells was tested after inoculation of C1-V13D into the thymus. FACS analyses using antibodies specific for different hematopoietic lineage markers, including T cell markers, was used to assess any phenotypic changes in C1-V13D cells following intrathymic transfers into allogeneic CBA/H mice and growth as a thymic tumour. The allogeneic model was used since C1-V13D cells can be readily distinguished by expression of the Class I H-2K^d determinant.

3.2 Materials and methods

3.2.1 Cell lines

The cell lines analysed are clones isolated following serial passage of C1-V13D through the thymus. Their derivation and maintenance have been described in Sections 2.2.1 and 2.3.6, respectively. All clones isolated following initial inoculation of C1-V13D intrathymically were designated as primary (1^o), and all second and third intrathymic passages as secondary (2^o) and tertiary (3^o), respectively.

3.2.2 Mice

CBA/H, DBA/2j and C57BL/6J X CBA/H (F1) mice were used. They were bred under specific pathogen free conditions in the John Curtin School of Medical Research, Canberra, Australia.

3.2.3 Intrathymic injections

Intrathymic inoculations were carried out as described in Section 2.2.6. Unless otherwise stated, thymic tumours referred to in this study resulted from intrathymic inoculation of $5x10^5$ C1-V13D cells or C1-V13D progeny cell lines.

3.2.4 FACS analysis

Cells were dissociated from the tumour mass and single cell suspensions were prepared as described in Section 2.2.7. For FACS analysis, cell suspensions were passed through sterile nylon mesh to remove cell clumps prior to staining. The antibodies used and the method for FACS analysis have been described in Section 2.2.4. For two colour FACS analysis, cells were first incubated with the primary test antibody, followed by FITC-sheep anti-mouse or sheep anti-rat IgG. The cells were then incubated for 30 minutes at 4°C with an isotype control antibody ($mIgG_1$; 19XE5 from Nowinski). This antibody was used to block unbound FITC-anti-Ig. The cells were then washed thrice with medium and incubated with biotinylated 30H-12, an antibody specific for Thy-1.2 (Serotec, Australia) and finally with streptavidin-R-phycoerythrin (PE) [Serotec, Australia]. The cells were washed twice with medium and resuspended in fixative (1% paraformaldehyde in normal saline/pH 7.4) before analysis.

Cell sorting and analysis were carried out using a FACS 440 Flow Cytometer (Becton Dickinson, Mountain View, CA) equipped with an Argon ion laser with 200mW output at a wavelength of 488nm. FITC emission was collected through a 530/20 Bandpass filter. Data was collected on a NEC 386/20 computer and analysed using WEFCS, a FACS data analysis package developed by Frank Battye (WEHI, Australia). Red blood cells and cell debris were gated out during analysis.

3.2.5 Northern Analysis

3.2.5A Preparation of RNA

The extraction of RNA was carried out according to the method of Chomczynski and Sacchi (1987), with modifications for small scale preparation. Ice-cold lysis buffer (4M guanidine thiocyanate, 0.025M tri-sodium citrate, 0.5% N-lauroyl sarcosine, 0.1M 2-mercaptoethanol/pH 7.0) was added to 10^6 pelleted cells. The mixture was vortexed vigorously for about 10 seconds, placing on ice between vortexing. Ice-cold 2M sodium acetate/pH 4.0 was added to a final concentration of 10% and the mixture was vortexed again. An equal volume of water-saturated phenol was added and briefly mixed, followed by a 5X volume of chloroform:isoamyl alcohol (24:1). The mixture was vortexed again and centrifuged at 4°C for 5 minutes at 15,800 x *g* in a microcentrifuge. The aqueous phase was transferred to a new tube and the RNA was precipitated with an equal volume of isopropanol at room temperature for 15 minutes. One to two ml of 4M lithium chloride was added to the RNA pellet to solubilise polysaccharides. The RNA was again pelleted at 15,800 x *g* for 30 minutes at 4°C, dissolved in 500µl TE-SDS (0.01M Tris-HCl/pH 7.5, 0.001M EDTA/pH 8.0 and 0.5% SDS) and re-extracted with an equal volume of chloroform:isoamyl alcohol. The aqueous phase was recovered after brief centrifugation to separate the phases. One-tenth volume of 2M sodium acetate/pH 4.0 was added, followed by an equal volume of isopropanol. The RNA was stored at -20°C under propanol, or pelleted at 4°C, redissolved in an appropriate amount of DEPC-treated ddH_20 and stored at -70°C in small aliquots.

The method of Davis *et al.* (1986b) was used for the extraction of RNA from liver, thymus and spleen. Tissue ($\leq$0.5g) was snap frozen in GITC buffer (47.3% guanidine isothiocyanate, 0.025M sodium acetate/pH 6.0, 0.1% 2-mercapthanol) using liquid nitrogen as the freezing medium. The tissues were homogenised immediately and the homogenate was stored at -70°C until required. A volume of $\leq$3.3ml of homogenate was layered on a cushion of 1.7ml DEPC-treated caesium chloride (CsCl) buffer (96% CsCl, 0.025M sodium acetate/pH 6.0) and ultracentrifuged at 149,000 x *g* using a SW55Ti rotor for 21 hours at 20°C in a Beckman L7 ultracentrifuge (Beckman Instruments Inc., USA).

The GITC layer and interface were removed after ultracentrifugation with a pasteur pipette and the CsCl cushion was decanted. Tubes were drained for 10 minutes and excess liquid was removed with a sterile Eppendorf tip. The RNA pellet was resuspended in 270µl DEPC-treated ddH_2O after cutting off the top part of the tube at a level approximately 1cm from the RNA pellet to prevent the collection of contaminating DNA. To this was added 3M sodium acetate/pH 5.6 to a final concentration of 10%. The RNA was precipitated with a 3.5X volume of ice-cold ethanol at -70°C. When needed, it was pelleted, washed twice with ethanol, dried and resuspended in an appropriate amount of DEPC - treated ddH_2O.

3.2.5B Electrophoresis of RNA and analysis

RNA was electrophoresed using denaturing formaldehyde gels as described by Sambrook *et al.* (1989). Samples were heated at 65°C in sample loading buffer (8% Ficoll, 0.02% bromophenol blue, 0.04% xylene cyanol) and denaturing cocktail (50% formamide, 17.5% formaldehyde, 0.01% MOPS, 0.05% ethidium bromide) for 10 minutes prior to loading. Agarose (Sigma, Type II) gels, containing 3.5 parts agarose, 1 part formaldehyde and 1.1 part 5X MOPS (0.2M MOPS, 0.05M sodium acetate, 0.01M EDTA/pH 7.0) were used. All equipment and solutions for RNA analysis were autoclaved immediately before use or treated with DEPC where autoclaving was not possible. After electrophoresis, gels were soaked (20°C, 60 mins) in 20X SSC (3M NaCl, 0.3M tri-sodium citrate/pH 7.0) before transferring to Hybond N^+ membranes (Amersham, Australia). They were then baked under vacuum at 80°C for 2 hours and stored in sealed plastic bags at -20°C. Preparation of ^{32}P-labelled probe and detection of specific nucleic acid sequence were carried out as described in Section 2.2.8D & E. The TCR-Cβ probe used for this study was provided by C. Okada (Stanford University). It is a 434bp *Eco*RI/*Hind*III fragment cloned into the pSPT672 plasmid vector and comprises the constant region of the T cell receptor β (TCR-Cβ) gene. It cross hybridises with the Cβ1 and Cβ2 genes.

3.3 Results

3.3.1 FACS analysis of thymic tumours: delineation of tumour subpopulations by gating

C1-V13D cells were initially transplanted intrathymically into syngeneic DBA/2j mice. FACS analysis on cells which constituted developing thymic tumours indicated that C1-V13D cells had differentiated and acquired mature T cell markers during the period of thymic replication (data not shown). However, it was not possible to conclusively distinguish C1-V13D cells from syngeneic DBA/2j host thymocytes either by size or using the available antibodies.

C1-V13D proliferated in the thymus of allogeneic CBA/H mice and progressively became more tumorigenic (Chapter 2). This transfer system into irradiated allogeneic hosts provided an alternative system to study the differentiative capacity of C1-V13D. C1-V13D cells could be distinguished from host CBA/H thymocytes on the basis of high Forward Scatter as well as expression of high levels of H-2K^d, B220, CD44, and the RadLV gp70 envelope protein. The anti-H-2K^d antibody, 34-1.2, is cross-reactive with different class I molecules (Ozato *et al.*, 1982) but not with H-2K^k, which is expressed by CBA/H thymocytes (Fig. 1). By gating the whole tumour population according to low and high 90^o Scatter (Figs. 2a & b), two and three subpopulations, respectively could be observed (Figs. 2c & d). This distribution of cell subpopulations by 90^o Scatter gating was seen in all thymic tumours analysed, including those from syngeneic DBA/2j mice and C57BL/6J X CBA/H (F1) mice (data not shown).

The subpopulations A and 3, observed with both high and low 90^o Scatter comprised small cells (Figs. 2c and 2d). These subpopulations do not appear to express any of the T cell markers tested. In a normal unirradiated CBA/H mouse, this population of 'marker negative' is barely detectable (Fig. 1). Since thymic tumours developed only in irradiated mice, the time frame required for normal thymic reconstitution following sublethal irradiation (4Gy) was analysed. This could be determined by FACS analysis and by the presence of the immature 'marker negative' subset. Full thymic reconstitution was indicated by an absence of 'marker

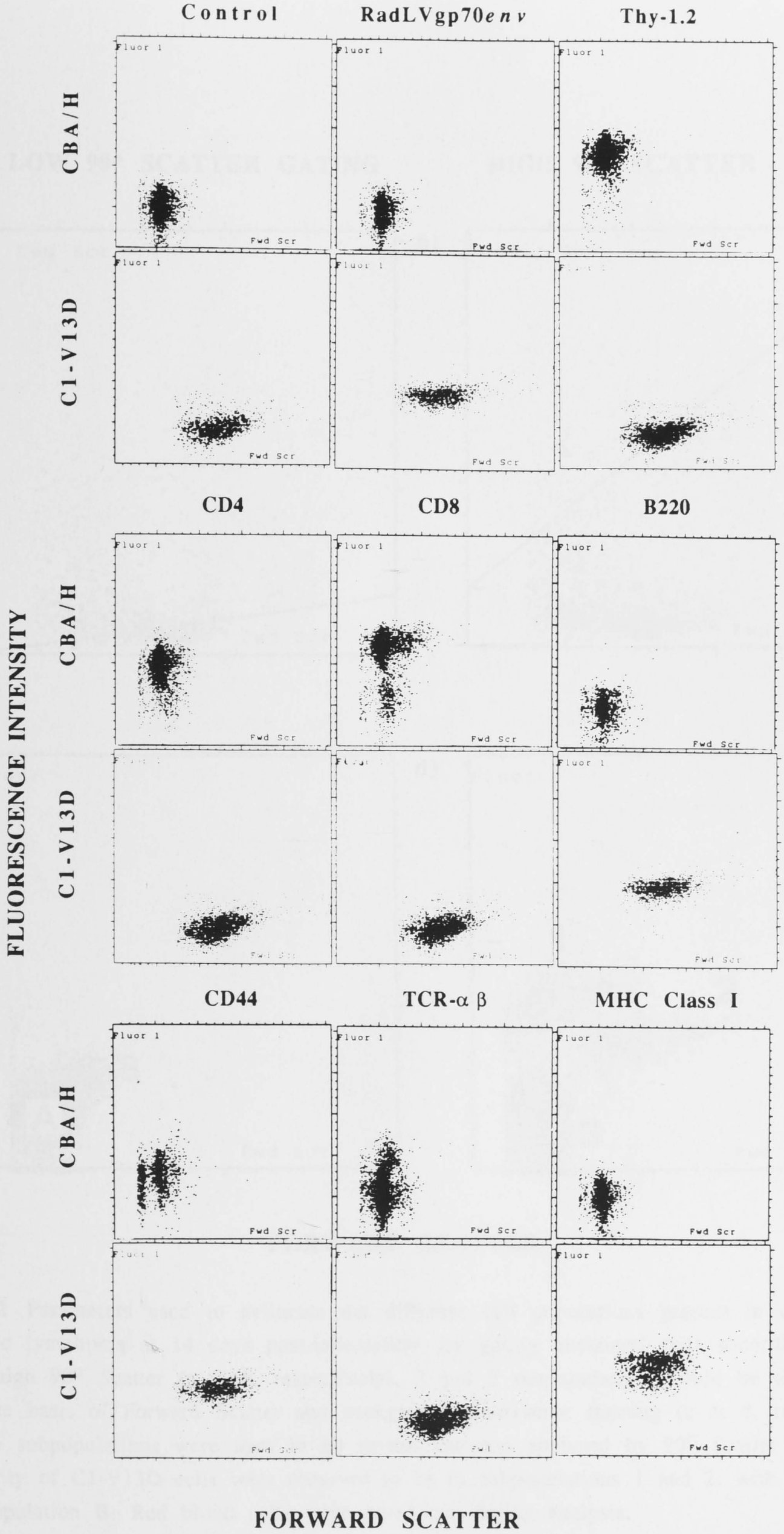


Fig. 1 FACS profile of C1-V13D compared to normal CBA/H thymocytes. C1-V13D can be distinguished from CBA/H thymocytes by the difference in size (indicated by Forward Scatter) and expression of B220, CD44 and the binding of the 34-1.2 antibody specific for MHC Class I H-2K^{d}.

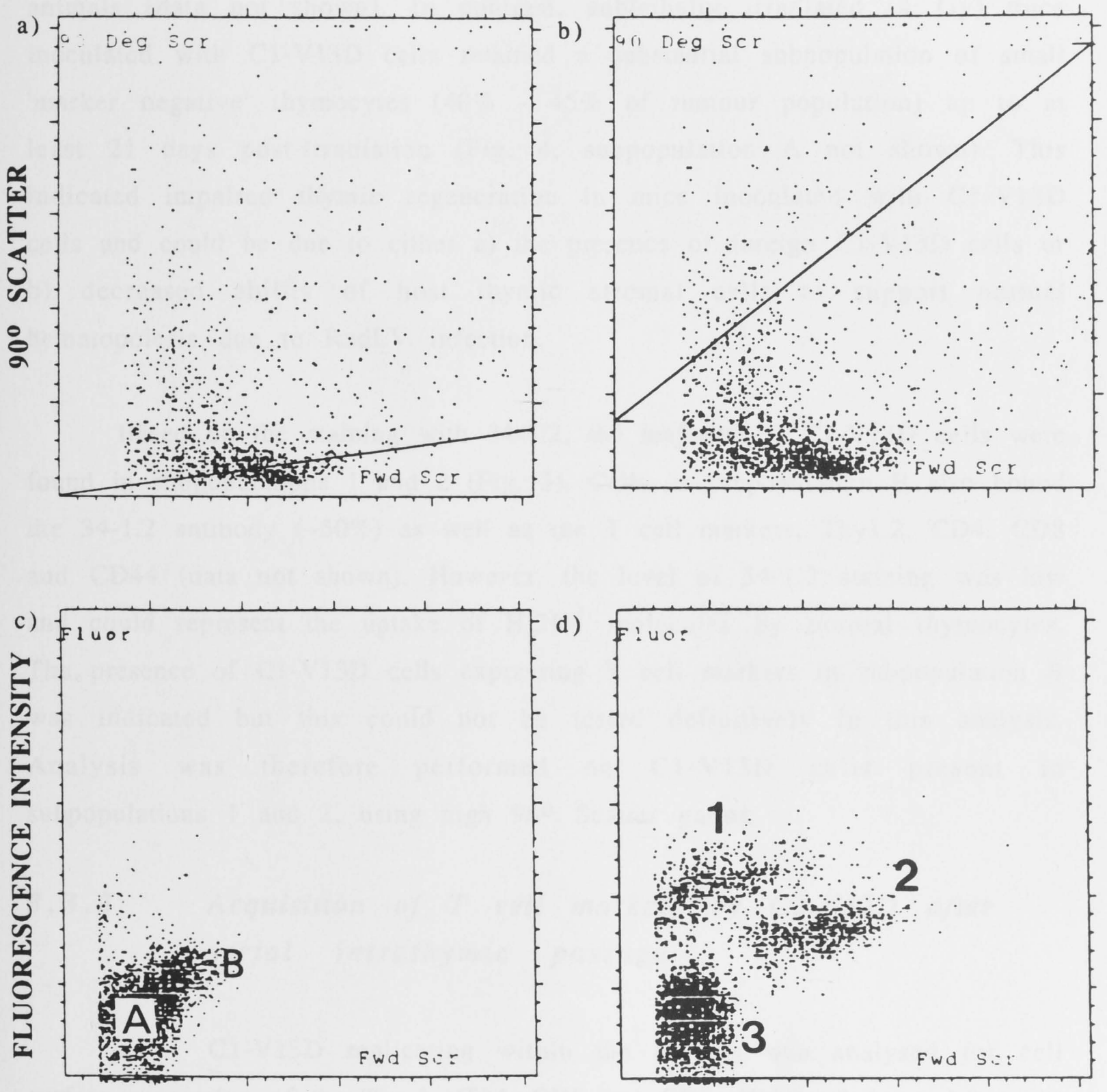


Fig. 2 Parameters used to delineate the different cell populations present in a typical thymic lymphoma at 14 days post-inoculation. By gating unstained cells according to low and high 90° Scatter (a & b, respectively), 2 and 3 subpopulations could be distinguished on the basis of Forward Scatter and background fluorescent staining (c & d, respectively). These subpopulations were seen in all thymic tumours analysed by 90° Scatter gating. The majority of C1-V13D cells were observed to be in subpopulations 1 and 2, with some in subpopulation B. Red blood cells were gated out during analysis.

negative' cells, similar to that observed in the thymus of unirradiated mice. The time taken for the recovery of normal thymus cell composition was found to vary between 11 and 14 days in most uninoculated, irradiated animals (data not shown). In contrast, sublethally irradiated (4 Gy) mice inoculated with C1-V13D cells retained a substantial subpopulation of small 'marker negative' thymocytes (40% - 45% of tumour population) up to at least 21 days post-irradiation (Fig. 4, subpopulation A not shown). This indicated impaired thymic regeneration in mice inoculated with C1-V13D cells and could be due to either a) the presence of foreign C1-V13D cells or b) decreased ability of host thymic stromal cells to support normal hematopoiesis due to RadLV infection.

Based on the staining with 34-1.2, the majority of C1-V13D cells were found in subpopulations 1 and 2 (Fig. 3). Cells in subpopulation B also bound the 34-1.2 antibody (~50%) as well as the T cell markers, Thy1.2, CD4, CD8 and CD44 (data not shown). However, the level of 34-1.2 staining was low and could represent the uptake of H-2K^d molecules by normal thymocytes. The presence of C1-V13D cells expressing T cell markers in subpopulation B was indicated but this could not be tested definitively in this analysis. Analysis was therefore performed on C1-V13D cells present in subpopulations 1 and 2, using high 90^o Scatter gating.

3.3.2 Acquisition of T cell markers by C1-V13D after serial intrathymic passage

C1-V13D replicating within the thymus was analysed for cell surface expression of the Thy-1, CD4, CD8 and CD3-ε/TCR-αβ determinants at different times post-inoculation. Analysis was difficult at 4 to 7 days post-inoculation as C1-V13D cells were present in numbers equivalent to only about 5-10% of the input cell dose. By 10 days, however, cell numbers had increased and C1-V13D cells were readily detectable by FACS analysis. Most analyses were performed when the hosts were moribund. This varied between 14 and 21 days post-inoculation.

A high 90^o Scatter gate was used to delineate C1-V13D cells for analysis in view of evidence that the majority of C1-V13D cells were found in subpopulations 1 and 2 (Fig. 3). T cell markers such as Thy-1, CD4 and CD8

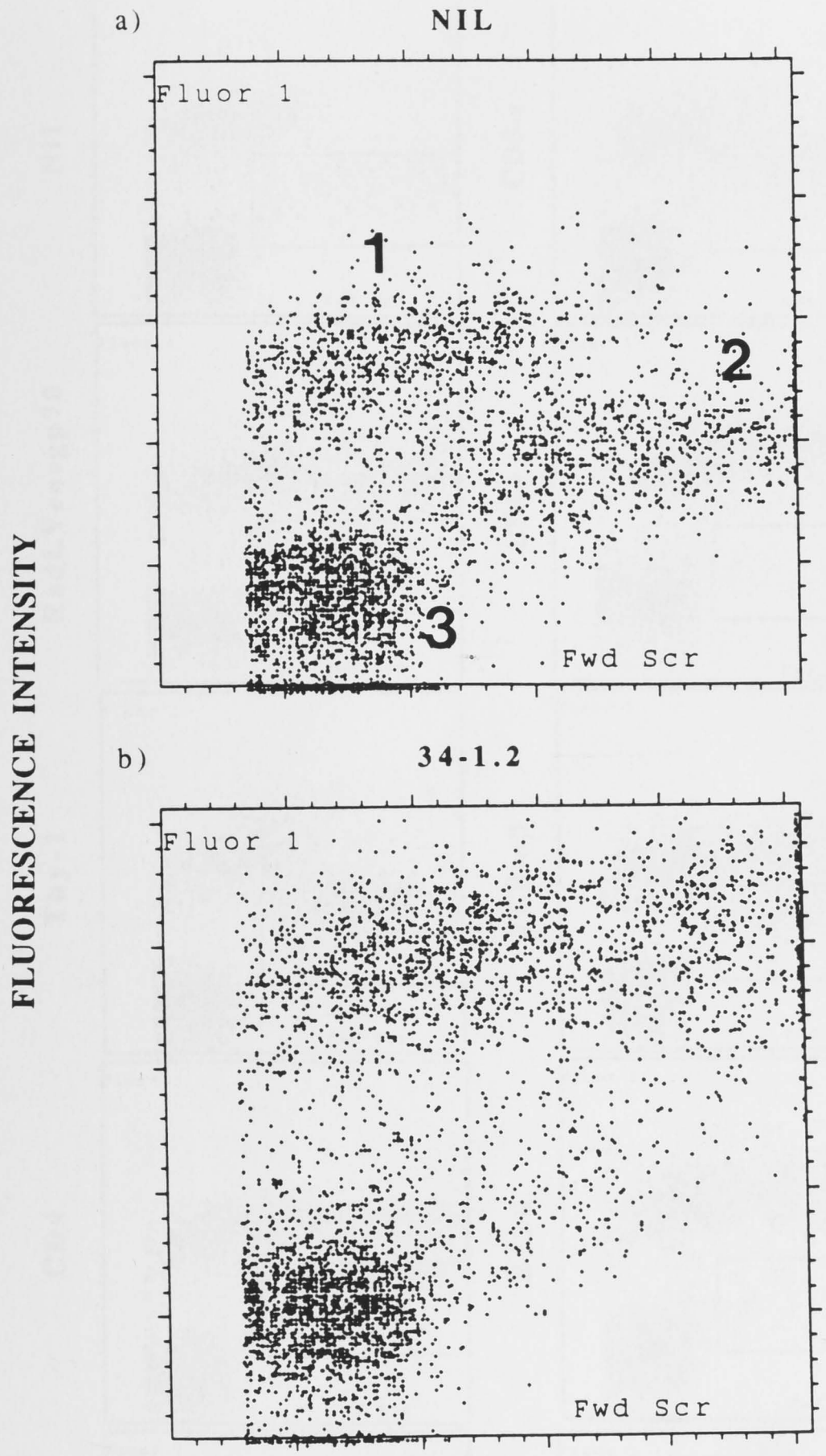


Fig. 3 FACS profile of ET4-D(T1), a 2° thymic tumour at 15 days post-inoculation of ET4-D, a 1° clone. High 90° Scatter gating was used to delineate cells. (a) Three cell populations can be distinguished on the basis of Forward Scatter and background fluorescence staining (control staining in which medium was used in place of specific antibody). (b) Specific staining using 34-1.2 (anti-H-2K^d). The majority of cells in populations 1 and 2 bound the 34-1.2 antibody, indicating their C1-V13D origin.

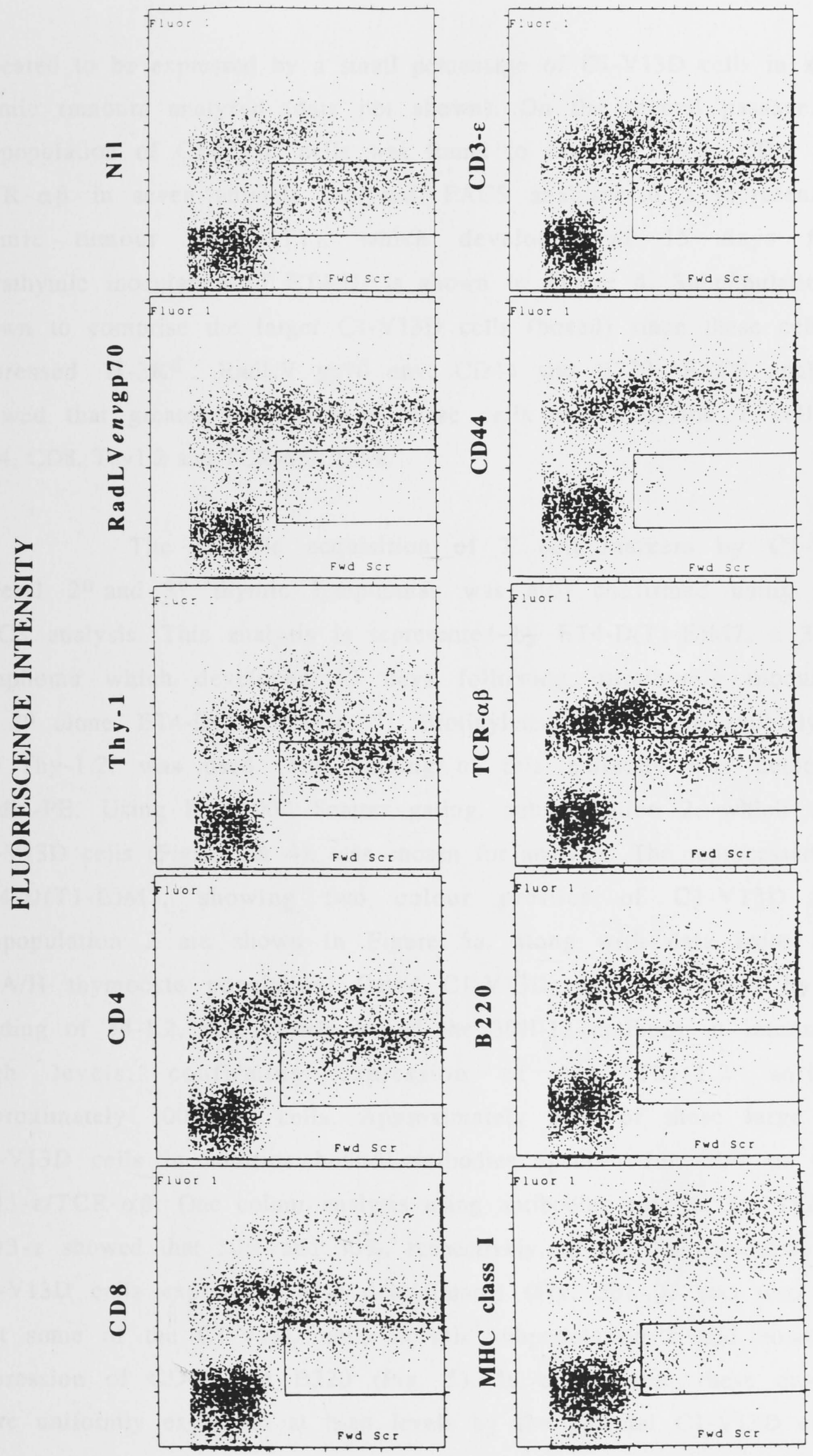


Fig. 4 High 90° Scatter profile of ET4-D(T1), a 2° thymic tumour at 15 days post-inoculation. Large C1-V13D cells were boxed for clarity. The acquisition of T cell markers (Thy-1, CD4, CD8, CD3-ε/TCR-αβ) by a subpopulation of C1-V13D cells is clearly seen.

appeared to be expressed by a small percentage of C1-V13D cells in all the 1^{o} thymic tumours analysed (data not shown). On the second passage, a clear subpopulation of C1-V13D cells was found to express Thy-1, CD4, CD8 and TCR-$\alpha\beta$ in seven animals analysed. FACS analysis of a representative 2^{o} thymic tumour ET4-D(T1), which developed at 15 days following intrathymic inoculation of ET4-D, is shown in Figure 4. Subpopulation 2 was shown to comprise the larger C1-V13D cells (boxed) since these cells clearly expressed H-$2K^{d}$, RadLV gp70 env, CD44 and B220. FACS analysis also showed that greater than 30% of these cells expressed the T cell markers CD4, CD8, Thy1.2 and TCR-$\alpha\beta$/CD3-ε.

The specific acquisition of T cell markers by C1-V13D in several 2^{o} and 3^{o} thymic lymphomas was also confirmed using 2 colour FACS analysis. This analysis is represented by ET4-D(T1-E)M7, a 3^{o} thymic lymphoma which developed 13 days following intrathymic inoculation of the 2^{o} clone, ET4-D(T1-E) [Fig. 5]. Biotinylated 30H-12, an antibody specific for Thy-1.2, was used. The binding of this antibody was detected with avidin-PE. Using high 90^{o} Scatter gating, subpopulation 2, which comprised C1-V13D cells (Figs. 2 & 4), was chosen for analysis. The reprocessed data for ET4-D(T1-E)M7, showing two colour profiles of C1-V13D cells in subpopulation 2 are shown in Figure 5a, along with data from a normal CBA/H thymocyte population. Gated C1-V13D cells, identified by uniform binding of 34-1.2, also clearly bound the 30H-12 antibody at intermediate to high levels, confirming expression of the Thy-1.2 antigen by approximately 100% of cells. Approximately 50% of these large Thy1.2^{+} C1-V13D cells in addition bound antibodies specific for CD4 or CD8 and CD3-ε/TCR-$\alpha\beta$. One colour analysis using antibodies specific for TCR-$\alpha\beta$ and CD3-ε showed that 50% and 30%, respectively, of this subpopulation of large C1-V13D cells expressed these determinants (Fig. 5b). It was also observed that some of the C1-V13D cells in this subpopulation 2 had downregulated expression of CD44 and B220 (Fig. 5). In comparison, these determinants were uniformly expressed at high levels by the parental C1-V13D cells (Fig. 1).

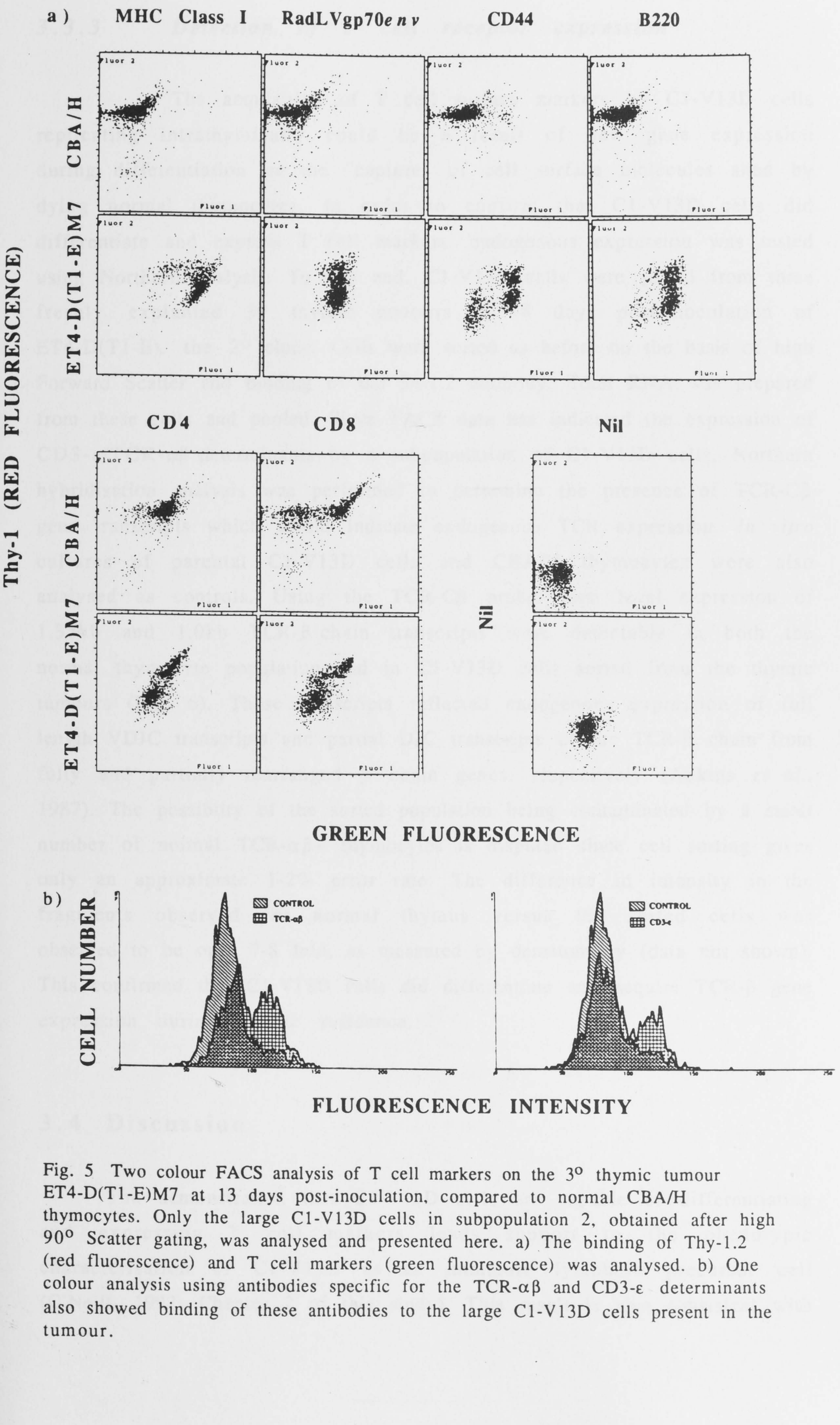


Fig. 5 Two colour FACS analysis of T cell markers on the 3° thymic tumour ET4-D(T1-E)M7 at 13 days post-inoculation, compared to normal CBA/H thymocytes. Only the large C1-V13D cells in subpopulation 2, obtained after high 90° Scatter gating, was analysed and presented here. a) The binding of Thy-1.2 (red fluorescence) and T cell markers (green fluorescence) was analysed. b) One colour analysis using antibodies specific for the TCR-αβ and CD3-ε determinants also showed binding of these antibodies to the large C1-V13D cells present in the tumour.

3.3.3 Detection of T cell receptor expression

The acquisition of T cell surface markers by C1-V13D cells replicating intrathymically could be a result of new gene expression during differentiation or the "capture" of cell surface molecules shed by dying normal thymocytes. In order to confirm that C1-V13D cells did differentiate and express T cell markers, endogenous expression was tested using Northern analysis. To this end, C1-V13D cells were sorted from three freshly explanted 3^o thymic tumours at 14 days post-inoculation of ET4-D(T1-E), the 2^o clone. Cells were sorted as before on the basis of high Forward Scatter and binding of the 34-1.2 antibody. Total RNA was prepared from these cells and pooled. Since FACS data has indicated the expression of CD3-ε/TCR-αβ determinants by a subpopulation of C1-V13D cells, Northern hybridisation analysis was performed to determine the presence of TCR-Cβ gene transcripts which would indicate endogenous TCR expression. *In vitro* cultures of parental C1-V13D cells and CBA/H thymocytes were also analysed as controls. Using the TCR-Cβ probe, high level expression of 1.35kb and 1.0kb TCR-β chain transcripts were detectable in both the normal thymocyte population and in C1-V13D cells sorted from the thymic tumours (Fig. 6). These transcripts reflected endogenous expression of full length VDJC transcripts and partial DJC transcripts of the TCR-β chain from fully and partially rearranged β chain genes, respectively (Adkins *et al.*, 1987). The possibilty of the sorted population being contaminated by a small number of normal TCR-αβ+ thymocytes is disputed since cell sorting gives only an approximate 1-2% error rate. The difference in intensity in the fragments observed for normal thymus versus the sorted cells was observed to be only 7-8 fold, as measured by densitometry (data not shown). This confirmed that C1-V13D cells did differentiate and acquire TCR-β gene expression during thymic residence.

3.4 Discussion

The demonstration that C1-V13D cells are capable of differentiating and expressing T cell markers lends support to the phenotypic characterisation of C1-V13D as an immature lymphoid precursor cell (O'Neill, 1992; Chapter 2 of this study). This result is also consistent with

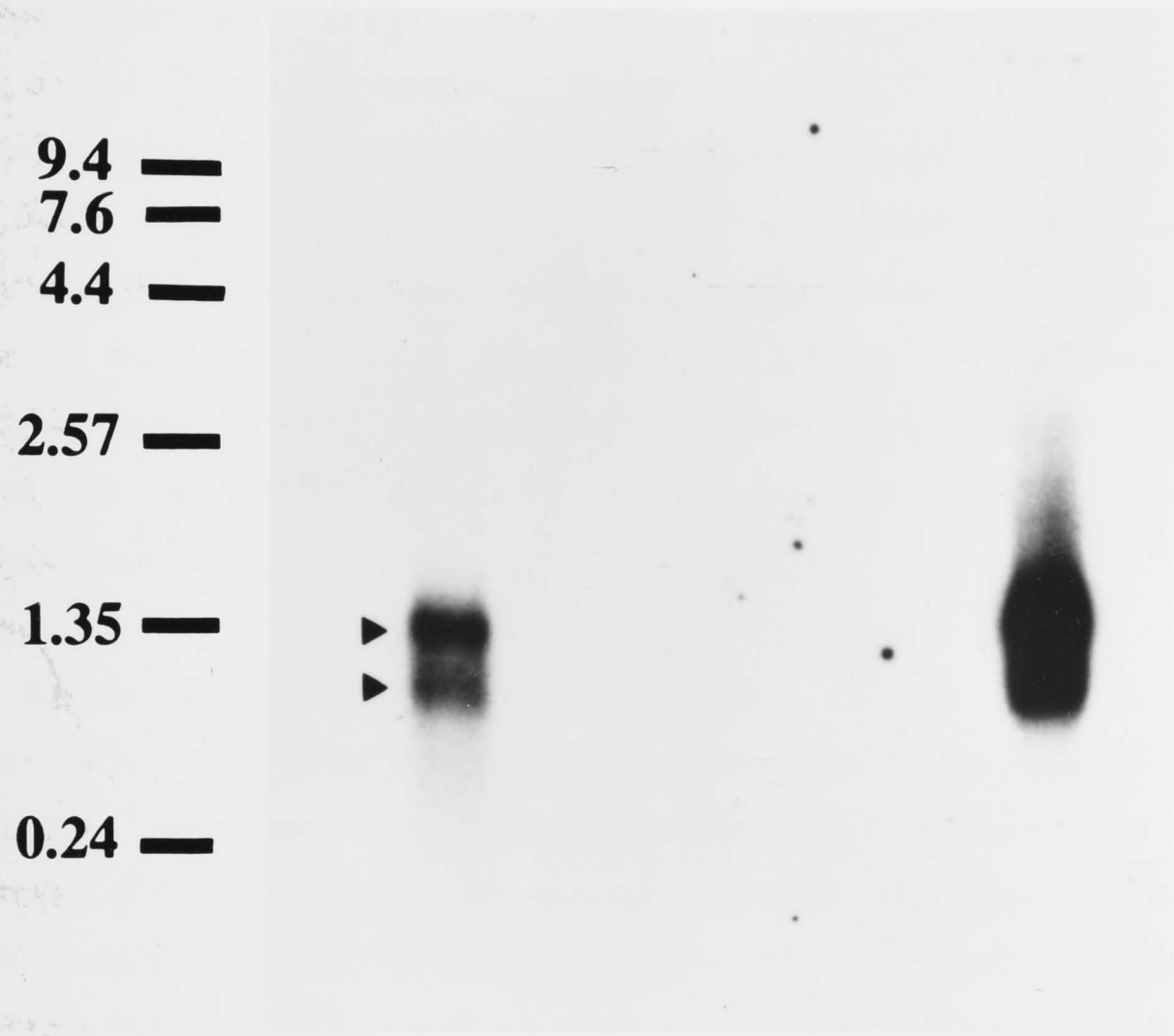


Fig. 6 Northern analysis showing TCR-β chain expression by C1-V13D cells reisolated from thymic lymphomas following third intrathymic passage. C1-V13D cells from 3 thymic lymphomas were sorted on the basis of high Forward Scatter and 34-1.2 antibody binding and their RNA was pooled. A total of 1.6μg RNA was obtained, which was used in this analysis. RNA (2μg) from normal CBA/H thymus and *in vitro* grown C1-V13D cells were included as controls. The Northern blot was analysed with a ^{32}P-labelled cDNA probe representing the TCR-Cβ gene. Specific signal was detected by autoradiography using X-ray film. RNA size markers (kb) are indicated.

previous studies which showed that RadLV preferentially infects immature hematopoietic cells in the bone marrow, spleen and thymus (Lieberman and Kaplan, 1976a). However, the migration of immature cells into the thymus and replication at this site appeared to be required for virus replication.

The development of T cell leukemia has been documented as a lengthy process involving a period of clonal cell selection and differentiation in the thymus (reviewed in Chapter 1). In experiments described here, an attempt was made to subject C1-V13D to the same selective pressure by serial passage through the thymus. Changes associated with differentiation and oncogenic progression were analysed using FACS analysis (this chapter) and *in vivo* studies (Chapter 2). Longer thymic residence was afforded by serial passages through the thymus. This seemed to result in a greater percentage of C1-V13D cells expressing mature T cell surface markers, and progressively tumorigenic progeny cells being selected as the cells replicated under the influence of the thymic microenvironment.

It has not been possible to determine by the methods used here whether C1-V13D cells have the capacity to differentiate into fully functional T cells and to emigrate to other peripheral lymphoid organs. It has been documented that 24 hours following the injection of double negative ($CD4^-CD8^-$) thymocytes, many of these cells become double positives ($CD4^+CD8^+$), with mature single positive cells evident in the periphery by 7 to 10 days (Scollay *et al.*, 1988). However, when C1-V13D cells were injected intrathymically, only thymic tumours developed and no other organs were involved at autopsy. This result was obtained for all cell doses and at each thymic passage. Three possible explanations could account for this apparent lack of infiltration by C1-V13D cells into the periphery : a) death of the host due to a thymic tumour occurred before C1-V13D could proliferate to detectable numbers in the peripheral organs, b) emigrating, mature C1-V13D were destroyed by the host immune system or c) C1-V13D cells could only undergo limited differentiation. So far, there have been no conclusive reports on whether RadLV-infected thymocytes could undergo normal differentiation. It is possible that RadLV may interfere with normal proliferative and differentiation events either

directly by insertional mutagenesis (reviewed in Section 1.5.3) or indirectly by partially impairing the ability of thymic stromal elements to support normal hematopoiesis. The latter effect has been described for the LP-BM5 MuLV, a murine AIDS virus. Tse *et al.* (1993) have shown that bone marrow stromal cultures infected with LP-BM5 could not establish the required microenvironment for normal hematopoiesis, resulting in depressed numbers of stem cell progenitors. In mice inoculated with C1-V13D, the 'marker negative' subpopulations "A" and "3" (Fig. 2) represent approximately 40% to 50% of the tumour population. These mice appear to have partially impaired thymocyte development and this could be due to either RadLV infection of thymic stromal elements or the presence of replicating C1-V13D cells within the thymus.

Despite clear expression of T cell markers by a substantial percentage of C1-V13D cells at the third intrathymic passage, C1-V13D cells isolated after *in vitro* culture of thymic tumours failed to show any evidence of differentiation. Initially, this was thought to be due to parental C1-V13D cells outgrowing their more "differentiated" counterparts in culture. However, when "differentiated" C1-V13D cells were sorted by Thy-1 expression and cultured in the absence of "parental" C1-V13D cells, Thy-1 expression was lost after only a week in culture (data not shown). The cells which grew up did not express any other T cell markers such as CD4, CD8 and TCR-$\alpha\,\beta$ as shown *in vivo,* but resembled the original parental C1-V13D in cell surface marker expression. It is possible that the Thy-1 expressing C1-V13D cells detected *in vivo* represent only a transient phenotypic change representative of the next step along the differentiation pathway and are incapable of replicating in the absence of an appropriate stimulus such as the thymic microenvironment. Similar results have been reported for the pre-B leukemic cell line G2 (Kamel-Reid *et al.,* 1992). This cell line was observed to express CD10 *in vivo*, but subclones isolated and propagated *in vitro* were shown to be $CD10^-$. These became $CD10^+$ again when they proliferated in the *scid* mouse thymus. The expression of CD10 on G2 cells was shown to be inducible and was maintained during cell replication in the thymus, but this expression was consistently lost upon culture. The dependence on physiological inducers in the thymic microenvironment for maintenance of determinant expression is implied in both studies.

Chemicals or growth factors have been used successfully to induce differentiation in leukemic cell lines along myeloid and erythroid lineages (Friend *et al.*, 1971; Sachs, 1978). Tumour promoting phorbol esters have been used successfully to induce differentiation in some human leukemic lines (Nagasawa & Mak, 1980; Delia *et al.*, 1982). Many attempts have been made to induce differentiation of C1-V13D *in vitro* with various stimuli including phorbol esters and growth factors but without success (O'Neill, personal communication). Within the limits of the model described here, data has been obtained which indicate that RadLV-immortalised 'targets' are capable of differentiation and can express T cell surface markers while replicating under the influence of the thymic microenvironment.

The inability of C1-V13D cells to retain expression of T cell markers after explantation from the thymus does not permit a definite association between differentiation and oncogenic progression to be made in this model. However, in view of the fact that differentiation is by itself a very complex process, it is possible that the increased tumorigenicity seen in C1-V13D after intrathymic passage may be due to genetic changes associated with early differentiative events having no correlation with the T cell surface markers analysed here. These early events could be a necessary prerequisite for further differentiation and stable expression of mature T cell surface markers such as Thy-1, CD4 and CD8. Genetic changes due to retrovirus infection may have precluded full differentiation into functional, mature T cells.

SUMMARY

The development of T cell leukemia has been documented to involve a lengthy selection process in the thymus, which is also coupled with a shift towards a more mature T cell phenotype. Serial intrathymic passage of C1-V13D cells into allogeneic CBA/H mice has been shown to result in increased tumorigenicity of the C1-V13D cell line. C1-V13D cells were therefore analysed in this study using flow cytometry for changes in cell surface marker expression related to differentiation in the thymus. The data obtained indicated conclusively that C1-V13D cells expressed the T cell markers, Thy-1.2, CD4 and CD8 and TCR-αβ/CD3-ε upon replication in the

thymic microenvironment, and supported earlier characterisation of C1-V13D as an immature lymphoid cell (Chapter 2). The expression of TCR-β gene transcripts was confirmed by Northern analysis on an isolated, sorted C1-V13D cell population. However, T cell marker expression was not stable and was consistently lost upon *in vitro* culture. Removal from the thymic microenvironment could account for this downregulation of marker expression. The increased tumorigenicity observed in C1-V13D after intrathymic passage did not appear to be related to stable expression of mature T cell surface markers analysed in this study but could be associated with genetic events in early T cell differentiation.

CHAPTER 4

Oncogenic progression: the significance of retroviral integration and recombination

4.1 Introduction

Retroviral recombination and reintegration events which occur after initial retroviral infection appear to play an important role in the process of transformation and oncogenic progression in T cell leukemia induced by slowly transforming retroviruses. These events have been associated with the generation of new leukemogenic viruses and the enhancement of genetic instability in the host genome via retroviral integration (reviewed in Sections 1.5.3 & 1.5.4).

A progressive increase in tumorigenic potential of C1-V13D has been demonstrated in progeny clones derived from thymic tumours following first, second and third passages intrathymically (Chapter 2). The C1-V13D cell line and the primary, secondary and tertiary clones derived from it were therefore used as a model for defining genetic events associated with transformation and oncogenic progression. This study is aimed at determining whether further retroviral integration and/or recombination, which could account for the observed increase in tumorigenicity, has occurred during serial intrathymic passage of C1-V13D cells. DNA from randomly selected primary, secondary and tertiary clones was digested with various restriction endonucleases and the retroviral integration pattern was analysed using probes subcloned from RadLV/V13, a virus cloned by Rassart *et al.* (1986). Changes in retroviral integration pattern which may correlate with an increase in tumorigenicity were sought. Since it has been shown that cell differentiation may be linked to the oncogenic process (Chapter 3), it is anticipated that genetic changes which can be associated with an

increase in tumorigenic potential, such as further retroviral integration events, could occur proximal to genes related to the differentiation of early lymphoid cells. Such a process would be driven by the strong selective pressure for cell replication within the thymic microenvironment.

4.2 Materials and methods

4.2.1 Cell lines

The origin, derivation and maintenance of cell lines has been described in Sections 2.2.1 and 2.3.6.

4.2.2 Bacterial strains

The E. coli host strains used were JPA101 (*sup E*, *thi*, D(*lac-proAB*), *recA*, Tc^R(::Tn 10), *ton*, l^r, F'[*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]) and DH5α' (*sup*E44, D*lac*U169 [*f* 80*lacZ*ΔM15], *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1, F'[*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]) which were compatible with blue/white colour screening and ampicillin selection.

4.2.3 Electron microscopy

Cells were prepared for electron microscopy as described in Section 2.2.3.

4.2.4 Derivation of viral probes for Southern analysis

4.2.4A Preparation of vector and insert DNA for cloning

The fragments to be subcloned for use as probes were excised from the RadLV/V13 proviral genome, which had been previously isolated and cloned into the pBR322 vector by Rassart *et al.* (1986). Briefly, RadLV/V13 was digested with the appropriate enzymes and electrophoresed through 1% GTG SEAKEM agarose (FMC Bioproducts,

USA) to separate the desired fragments. These were excised and the DNA was extracted from the agarose using the Geneclean kit (Progen, USA).

The pGEM^R plasmid vectors (Promega Corporation, USA) were used for subcloning. They were dephosphorylated with calf intestinal alkaline phosphatase (Pharmacia, USA) to prevent self-ligation. Dephosphorylation was carried out according to Sambrook *et al.* (1989). Appropriately digested vector and viral fragments were ligated, usually in a 1:2 or 1:3 vector:insert molar ratio in ligase buffer (Promega, USA) containing 1 Weiss unit of T4 DNA ligase (Pharmacia, USA) for 16 to 18 hours at 12^{o}C for blunt end ligations, or 16^{o}C for cohesive end ligations.

4.2.4B Transformation by heat shock

E. coli cells for 'heat shock' transformation were made competent according to the method developed by Hanahan (1983). Briefly, the cells were grown to mid-exponential phase in 30ml to 100 ml of SOB medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 250mM KCl, 0.01M $MgCl_2$, 20mM $MgSO_4$) and chilled on ice. The cells were recovered by centrifugation at 4000 x *g* for 10 minutes at 4^{o}C. After completely draining away the last traces of media, the cell pellet was resuspended in 20ml ice-cold FSB buffer (10mM potassium acetate/pH 7.5, 45mM $MgCl_2.4H_20$, 10mM $CaCl_2$, 100mM KCl, 3mM hexamminecobalt chloride, 10% [v/v] glycerol) and kept on ice for 10 minutes. The cells were then pelleted, drained of buffer and resuspended again in 4ml ice-cold FSB buffer. Dimethyl sulphoxide (DMSO) was added to a final volume of 0.07% in 2 aliquots, allowing 15 minutes to chill on ice after the first DMSO addition. The cells were quickly dispensed into chilled sterile microfuge tubes, snap-frozen using liquid nitrogen and stored at -70^{o}C.

The procedure for heat shock transformation was carried out according to Sambrook *et al.* (1989). Prior to transformation, 200µl cells were thawed on ice for 10 minutes and then mixed with 25 to 50ng of the ligated vector/insert DNA. The DNA/cell mixture was incubated on ice for 45 to 60 minutes, heat shocked for 100 seconds at 42^{o}C, and chilled immediately on ice. Pre-warmed SOC medium (1% tryptone, 0.5% yeast

extract, 0.5% NaCl, 0.01M $MgCl_2$, 0.01M $MgSO_4$, 0.5% glucose/pH 7.0) was added to a final volume of 1ml. The cells were incubated at 37°C for 10 minutes without agitation, after which they were incubated for another 60 minutes at 37°C with gentle agitation at 150rpm in an incubator shaker (New Brunswick, USA). The transformed cells were pelleted, resuspended in 25µl LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and plated on LB agar plates (1.2% bacto-agar) containing 100µg/ml Ampicillin (Sigma, USA), 0.5mM IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma, USA) and 40µg/ml X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside; Boehringer Mannheim, Germany). X-gal was prepared as a 50mg/ml stock solution in N,N' dimethylformamide. The plates were inverted and incubated at 37°C for 18 hours. Six to eight white recombinant colonies from each ligation reaction were selected for restreaking twice before checking for clones bearing inserts of the correct size.

4.2.4C Transformation by electroporation

To prepare electrocompetent cells, 1 litre of cells was grown to exponential phase. The cells were chilled briefly on ice, harvested at 4000 x *g* for 10 minutes at 4°C and washed twice with 500ml to 1 litre of cold ddH_2O. They were then resuspended in 20ml of cold 10% glycerol and kept at 4°C on ice overnight. Cells were pelleted and resuspended in approximately 3ml (final volume) of cold 10% glycerol. The cells were aliquoted, snap frozen in liquid nitrogen and stored at -70°C.

DNA for transformation by electroporation was first precipitated by the addition of 0.1X volume 3M sodium acetate/pH 5.2 and 2.5X volume ethanol. The pellet was washed twice with ethanol, vacuum-dried and resuspended in 5µl ddH_20. Thawed electrocompetent cells (40µl) were mixed with 2µl DNA chilled on ice and placed in an ice-cold single use electroporation microcuvette with a 2mm electrode gap (Biorad, USA). The cuvette was set in position in the sliding cuvette holder and electroporation was carried out with the Gene Pulser (Biorad, USA) set at 2.5kV and 25µF capacitance and the pulse controller set at 200Ω resistance. The resulting pulse was 12.5 KV/cm with a time constant of 4.0 milliseconds. Immediately following the pulse, the cells were mixed

with 1ml pre-warmed SOC medium and incubated at 37°C with gentle agitation as for heat-shock transformation. Cells were then plated on selective LB agar.

4.2.4D Mini-scale plasmid preparation

Preparation of plasmid DNA on a small scale for quick analysis was carried out according to the method of Wilimzig (1985). The cell pellet of a 2ml overnight culture was resuspended in cold 250µl TELT buffer (4% Triton X-100, 0.0625M EDTA/pH 8.0, 0.05M Tris/pH 7.5, 0.1% LiCl) and chilled on ice for 5 minutes. A 25µl volume of freshly prepared aqueous solution of lysozyme (10mg/ml) was added. The mixture was boiled for 1 minute and centrifuged at 15,800 x *g* in a microfuge for 30 minutes at 4°C. The pellet (comprising bacterial chromosomal DNA and cell debris) was removed with a sterile toothpick and the plasmid DNA was precipitated with 2X volumes of 95% ethanol and pelleted immediately at 15,800 x *g* for 15 minutes. The DNA pellet was washed twice with 70% ethanol, vacuum dried and resuspended in 50µl of ddH_2O. Three to 5µl of the resuspended DNA was usually sufficient for a single restriction enzyme analysis.

4.2.4E Large scale plasmid preparation

A one-tenth volume of an overnight 5ml cell culture containing the desired recombinant plasmid was inoculated into 500ml LB medium and grown overnight at 37°C with vigorous agitation in an incubator shaker (approximately 300rpm). The cells were pelleted by centrifugation at 4000 x *g* for 15 minutes using a SA600 rotor in a Sorvall centrifuge (Du Pont Instruments, Australia) at 4°C, resuspended in 10ml of 25% sucrose/TE and maintained on ice. To this was added 1.5ml of freshly prepared lysozyme solution (20mg/ml in 0.25M Tris/pH 8.0) and 5ml of 0.5M EDTA/pH 8.0. The mixture was swirled intermittently on ice for 5 minutes. To this mixture, 15ml of Brij/DOC solution (0.01M Tris/pH 7.4, 0.001M EDTA/pH 8.0, 0.01% Brij 58, 0.4% sodium deoxycholate) was added rapidly, mixed quickly by inversion several times and left on ice until lysis had occurred. Cell debris and chromosomal DNA were pelleted by centrifugation at 39,000 x *g* using a SS-34 rotor for 1 hour at 4°C. The

clear supernatant was carefully decanted into a clean polypropylene tube and 3% w/v NaCl and 1/4 volume of 50% polyethylene glycol (PEG 6000) were added. The plasmid DNA/PEG complex was left on ice for at least 2 hours and pelleted by mild centrifugation. Dissolution of the pellet was facilitated by warming the solution to 65°C in 5ml TES (0.01M Tris/pH 7.4, 0.001M EDTA/pH 8.0, 0.58% NaCl). Caesium chloride (8.0g) and 0.6ml of ethidium bromide (10mg/ml) were added. The caesium chloride was dissolved by inversion and the mixture was left on ice for between 30 minutes and 1 hour. Under these conditions, PEG was displaced from the solution and formed a flocculent precipitate at the top of the tube. After centrifugation at 12,000 x *g* for 30 minutes at 4°C, the supernatant was decanted carefully into polyallomer ultracentrifuge tubes leaving the flocculent PEG precipitate adhering to the polypropylene centrifuge tube walls. The density of caesium chloride was adjusted to 1.6g/ml by addition of approximately 1.5ml of TES. Paraffin oil was added using a syringe fitted with a 21G needle and the tubes were balanced to within 50mg by the addition of caesium chloride (1.6g/ml). The tubes were ultracentrifuged in a Beckman L7 ultracentrifuge using a VTi65 rotor (Beckman Instruments Inc., USA) at 320,000 x *g* overnight at 18°C. The lower plasmid band was extracted with a side puncture just beneath it using a 1ml syringe fitted with a 19G needle. Ethidium bromide was removed by extracting the plasmid DNA thrice with an equal volume of water saturated butanol. The DNA was then dialysed against 1X TE buffer with 3 changes and its concentration was measured at OD_{260}.

4.2.5 Southern analysis

The procedure for Southern analysis, including hybridisation with specific DNA probes has been described in Section 2.2.8. The probes used were derived from RadLV/V13, a full length ecotropic retroviral clone. They are the LTR probe, a 500bp *Pst*I/*Kpn*I fragment, the gp70 *env* and p15E probes which were cloned as 800bp *Hpa*I/*Bam*HI and 850bp *Bam*HI/*Xba*I fragments, respectively, and the leader/*gag* (L) and *pol* probes, derived as 550bp *Pvu*I/*BstE*II and 600bp *Kpn*I/*Kpn*I fragments, respectively (Fig. 1). The Rad/LV gp70 *env* probe overlaps the murine ecotropic *env* specific probe as defined by Chattopadhyay *et al.* (1982). Restriction sites in RadLV/V13 were mapped

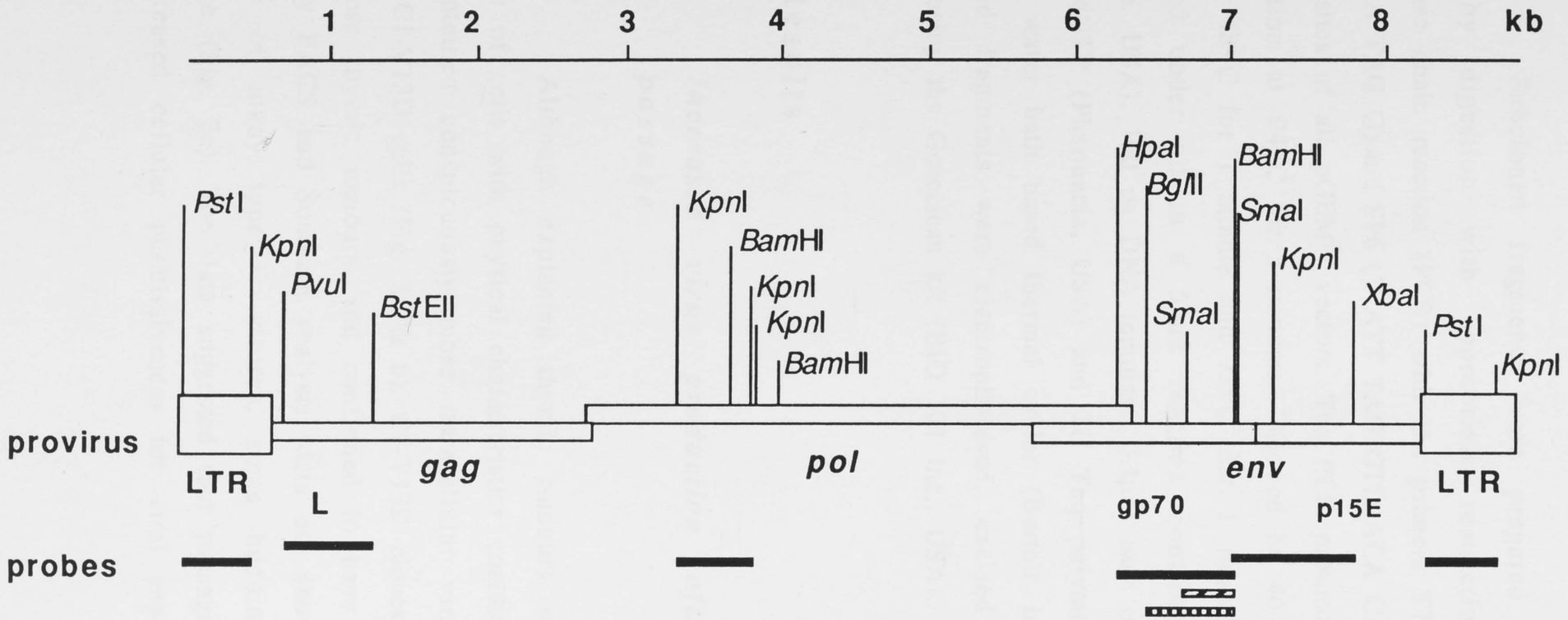


Fig. 1 Restriction map of the RadLV/V13 clone. Restriction sites were mapped by Rassart *et al.* (1986) and confirmed in this laboratory. DNA fragments used as probes are indicated by solid black lines. These were subcloned from pBR322/V13 into pGEM^{R} vectors. (▭) and (▬) represent the position of the AKR ecotropic *env* probes used by Chattopadhyay *et al.* (1982) and Moore & Chan (1982), respectively.

by Rassart *et al.* (1986) and also in this laboratory by Dr C. Jolly, and were found to be consistent with the complete nucleotide sequence of RadLV-pMOL52 (Merregaert *et al.*, 1987).

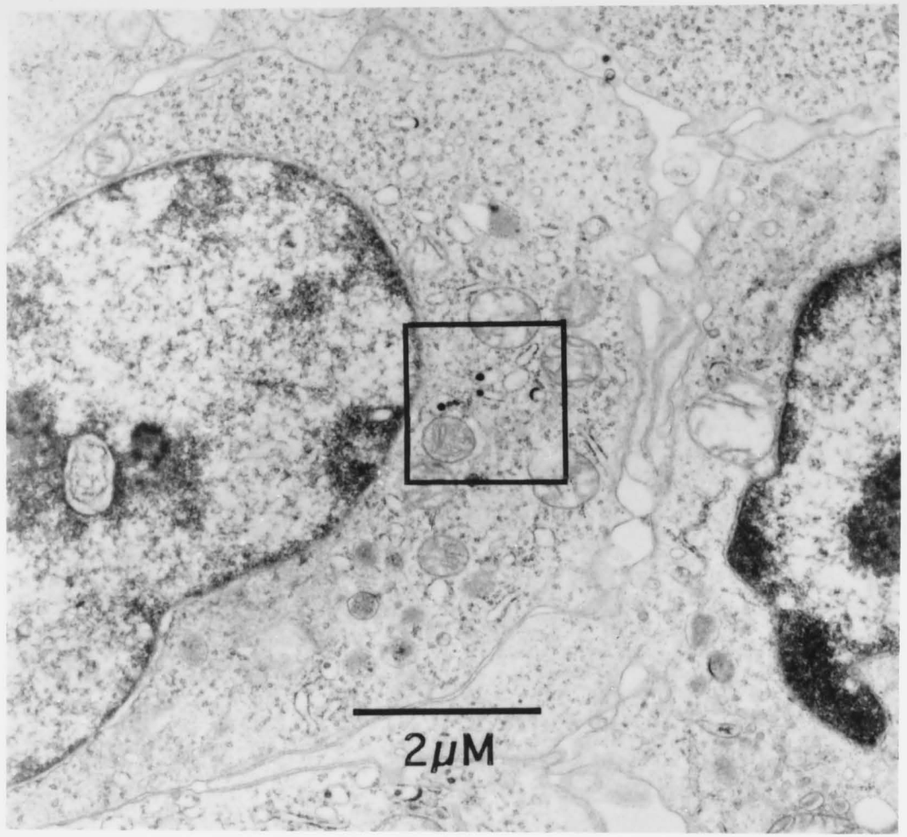
Subcloned fragments were prepared for use as probes either by digestion with appropriate restriction enzymes or by polymerase chain reaction (PCR) with the primers T7 (5' TAA TAC GAC TCA CTA TAG G) and SP6 (5' ATT TAG GTG ACA CTA TAG) that flank the cloning sites of all pGEM^R vectors. The PCR protocol comprised an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of 94°C for 1 minute, 48°C for 1 minute and 72°C for 1 minute. Amplification was performed under oil in a 50µl reaction containing 1X PCR buffer (Promega, USA), 100 pg DNA template, 0.1µM each of primers SP6 and T7, 0.2mM dNTP (Pharmacia, USA) and 2U Taq polymerase (Promega, USA) using a water bath based thermal cycler (Bartelt Instruments, Australia). Amplified fragments were electrophoresed, excised and extracted from the gel using the Geneclean kit (BIO 101 Inc., USA).

4.3 Results

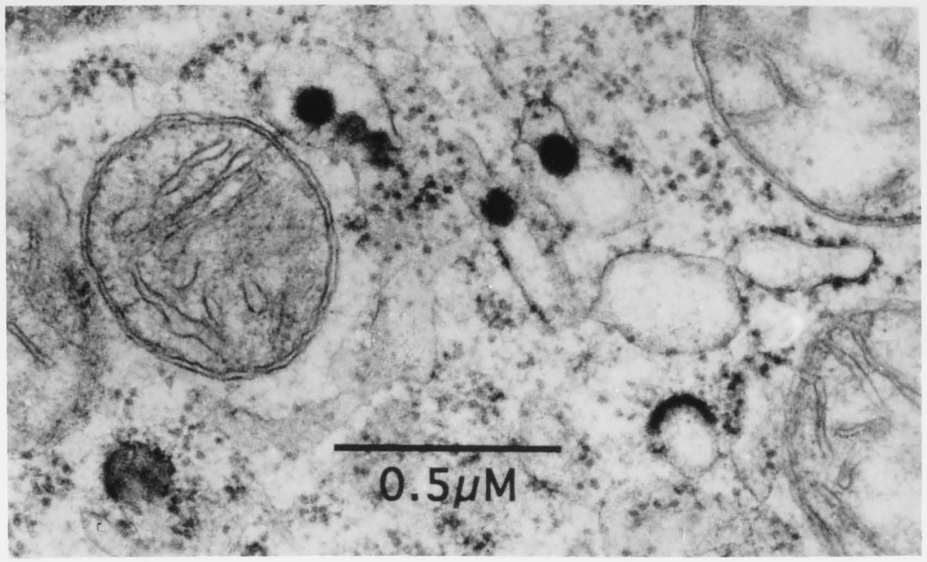
4.3.1 Increased virus production after intrathymic passage

Although explanted thymic tumours were observed to be made up of cells with physical characteristics common to C1-V13D cells, they contained conspicuously more intracellular viral particles than the original C1-V13D cells (Fig. 2a & b). C1-V13D clones established in culture from these thymic tumours, and confirmed to have originated from C1-V13D by FACS and Southern analysis (data not shown) also revealed the presence of many type C viruses, some budding from the plasma membrane (Fig. 2c). The data suggested that passage through the thymus had increased cellular permissiveness for viral replication.

a)



b)



c)

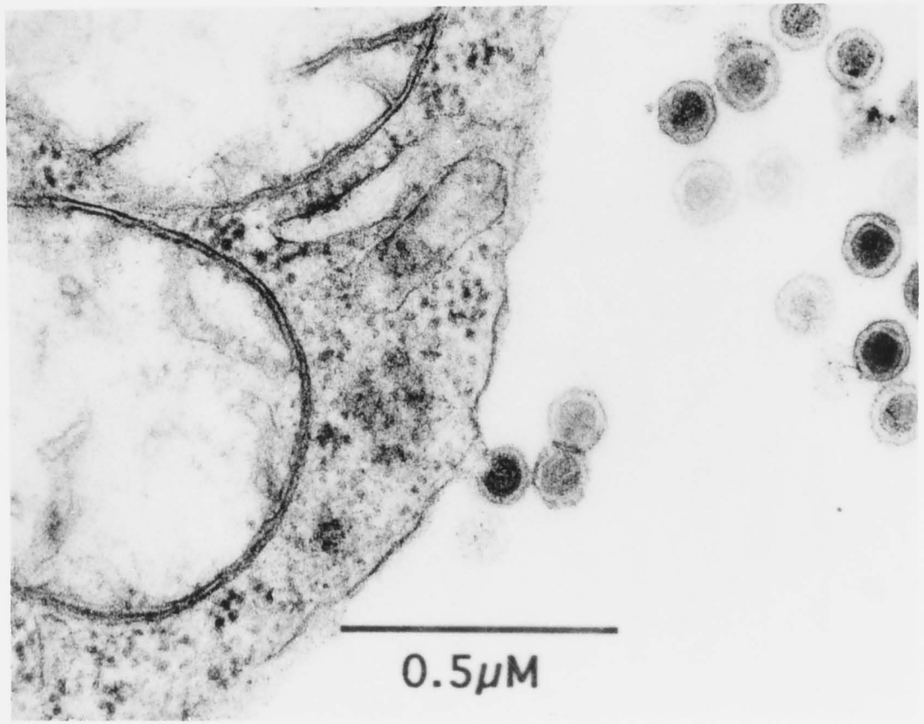


Fig. 2 Electron micrographs of (a) a 1° thymic lymphoma which resulted from intrathymic inoculation of C1-V13D and (b) a higher magnification of the boxed region showing the presence of intracellular virus particles. (c) One clone isolated following *in vitro* culture revealed numerous C-type viral particles, some of which can be seen budding from the plasma membrane.

4.3.2 *C1-V13D harbours numerous RadLV or RadLV-like proviruses integrated into its genome*

DNA from C1-V13D was compared with DNA prepared from syngeneic DBA/2j and allogeneic CBA/H mouse livers for the presence of any newly integrated RadLV-like viral genomes using the RadLV gp70 *env* probe, which is sufficiently specific for ecotropic envelope sequences (Chattopadhyay *et al.*, 1982; Merregaret *et al.*, 1987). DBA/2j mice carry only full length endogenous ecotropic RadLV or RadLV-like genomes, as indicated by *Pst*I and *Eco*RI digests (Fig. 3a & 3b). The single band was shown to represent at least two proviruses, one or both of which has an internal *Hind*III site (Fig. 3c). With this probe, no endogenous viruses were detectable in DNA from CBA/H mice, indicating that CBA/H mice do not carry any ecotropic RadLV or RadLV-like viruses. This result reinforces the suitability of the CBA/H mouse as a host strain in this study. C1-V13D was found to carry many newly integrated viruses in its genome compared to DBA/2j liver DNA (Fig. 3). *Pst*I digestions revealed the presence of four distinct viral fragments in C1-V13D which were approximately 9.0kb, 5.5kb, 2.5kb and 1.9kb in size (Fig. 3a). The smaller fragments (<6.6kb) could represent viruses with an internal *Pst*I site, or defective viruses. Multiple bands, some clearly smaller than the full length virus were also observed in *Eco*RI- and *Hind*III-digested C1-V13D DNA. Since the original RadLV/V13 clone does not carry any internal *Hind*III or *Eco*RI restriction sites, these smaller fragments represent either defective or recombinant viruses.

The less specific RadLV probes which hybridised with the *pol*, L/*gag* and p15E sequences also detected multiple retroviral bands in both CBA/H and DBA/2j liver DNA (Figs 4 & 5; p15E data not shown). In comparison with DBA/2j germline DNA, however, C1-V13D showed several new viral bands. These were detectable with each of the 3 probes using the three different restriction enzymes, *Pst*I, *Eco*RI and *Hind*III (Figs. 4 & 5, p15E data not shown).

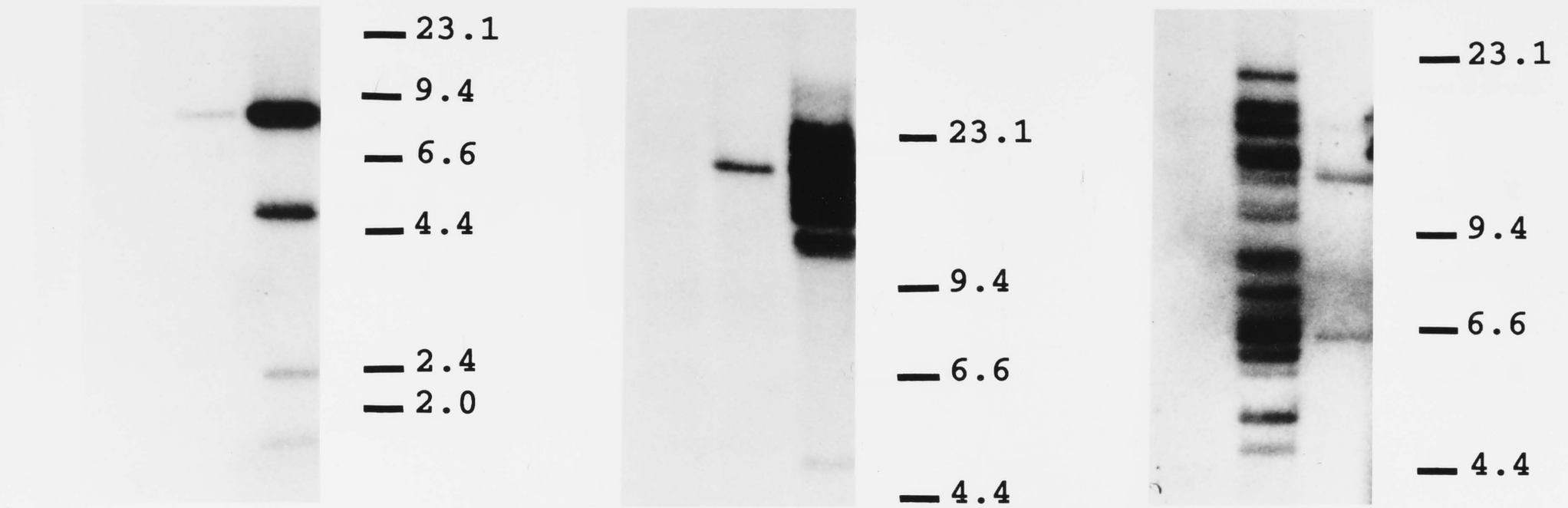


Fig. 3 Southern analysis showing the pattern of RadLV or RadLV-like proviral integrations in C1-V13D cells. DNA was digested with (a) *Pst*I, (b) *Eco*RI and (c) *Hind*III and analysed with the gp70 *env* probe. C1-V13D DNA was compared with germline DNA from CBA/H and DBA/2j liver (LV). DNA size markers (kb) are indicated.

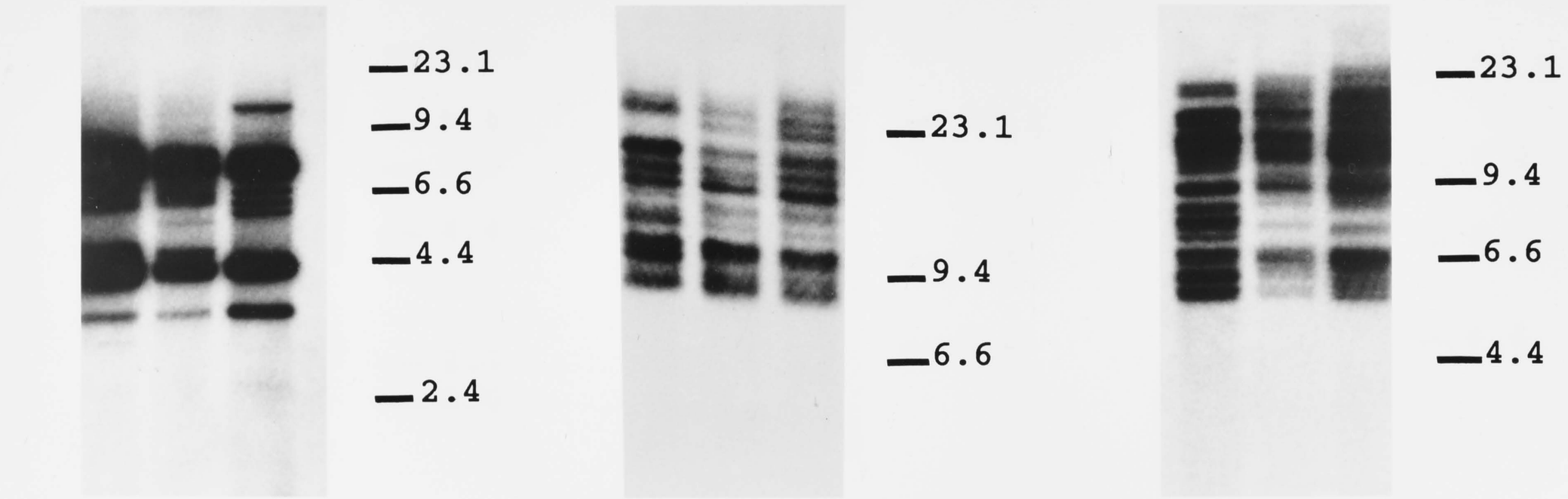


Fig. 4 Analysis of proviral integrations in C1-V13D genomic DNA digested with (a) *Pst*I (b) *Eco*RI and (c) *Hind*III using the *pol* probe. DNA from CBA/H and DBA/2j liver (LV) were included for comparison. DNA size markers (kb) are indicated.

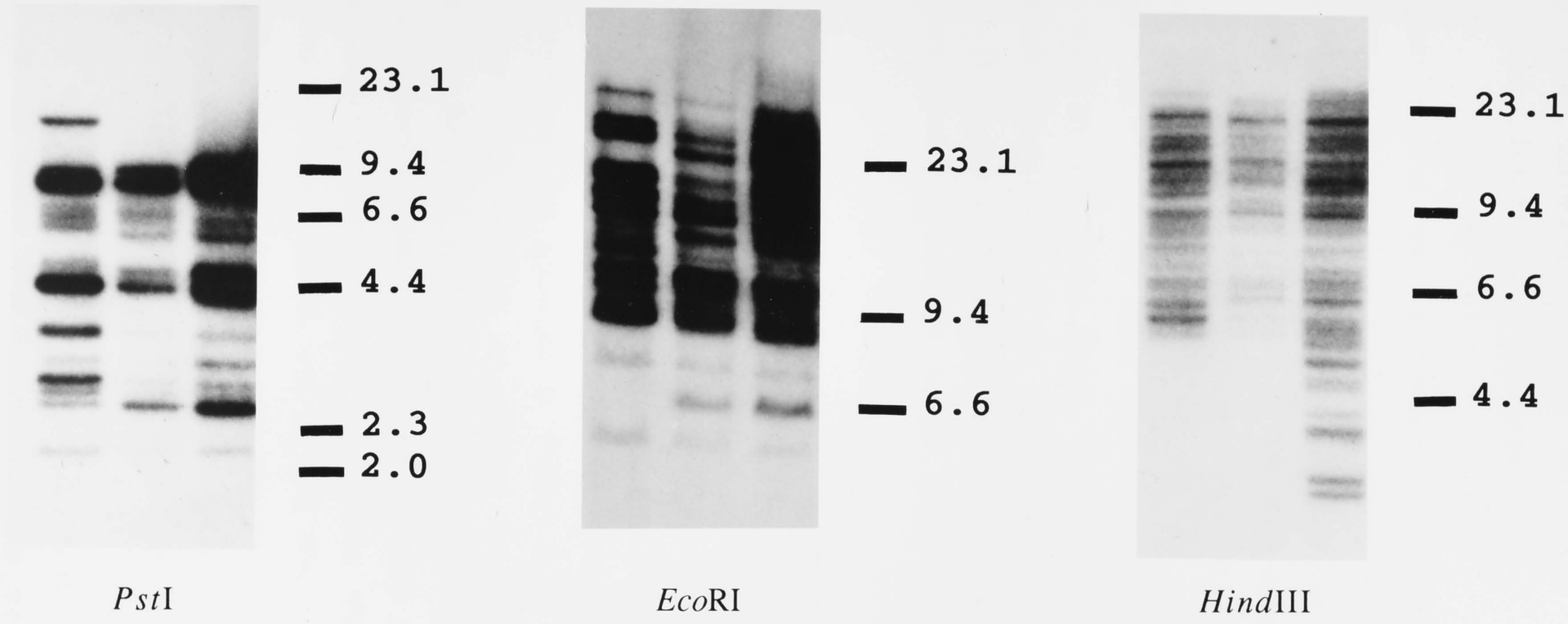


Fig. 5 Pattern of proviral integrations in C1-V13D cells detectable after digestion with 3 different enzymes. C1-V13D DNA was compared with syngeneic DBA/2j and allogeneic CBA/H liver DNA using the leader/*gag* (L) probe. DNA size markers (kb) are indicated.

4.3.3 Southern analysis revealed new retroviral events in some C1-V13D progeny cell lines derived after intrathymic passage

Southern analysis was used to determine whether new retroviral integration events had occurred in C1-V13D during the period of cell replication in the thymus. The leader/*gag* (L), *pol*, gp70 *env*, p15E and LTR probes were used to detect any changes in the viral integration patterns in C1-V13D and progeny cell lines. Three restriction enzymes were used to digest DNA, namely *Pst*I, *Eco*RI and *Hind*III. As mentioned previously, *Pst*I cuts the RadLV/V13 viral clone once within each LTR and can therefore be used to assess the length of integrated viral genomes. Both *Eco*RI and *Hind*III do not cut within the RadLV/V13 genome (Rassart *et al.*, 1986) and can therefore be used to assess the number of integration events and to provide a unique clonal integration pattern.

DNA preparations from randomly selected clones were compared. They were derived *in vitro* from primary, secondary and tertiary tumours which developed following intrathymic inoculation of C1-V13D cells. DNA was digested with *Pst*I, *Hind*III and *Eco*RI and analysed using all 5 viral probes. The data from nine primary, two secondary and four tertiary clones are presented in Figures 6, 7 & 8. Restriction analysis using *Pst*I detected no change in retroviral integration pattern, either with the LTR, L, p15E or the gp70 *env* probes (Fig. 6 & 7, some data not shown). A 5.5kb viral band in C1-V13D and in progeny cell lines was detected with the gp70 *env* probe (Fig. 7, arrowed). This fragment also hybridised with the *pol*, L, LTR (Fig. 6a) and p15E probes, albeit poorly with the latter (data not shown). A likely explanation is that this fragment is the 5'-end of a virus with a *Pst*I site within the p15E region covered by the probe, or it is a defective virus with a deletion involving the p15E region. The smaller 2.4kb gp70 *env* positive viral band (Fig. 7) has no coincident *pol* or L/*gag* sequences which could be detected with the probes (Fig. 6), and may also represent a defective virus carrying only the RadLV gp70 *env* and p15E sequences or the 3'-end of a virus with an internal *Pst*I site different from the virus discussed above.

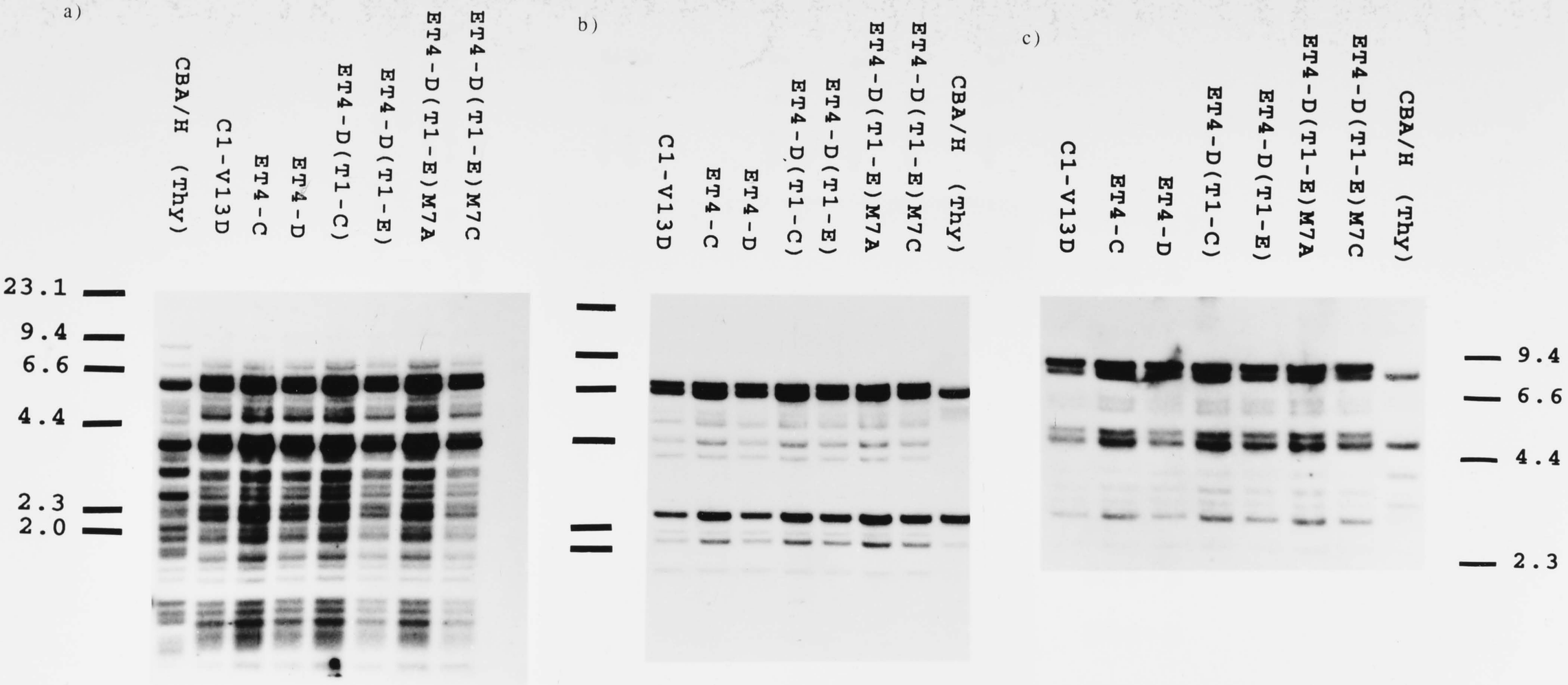


Fig. 6 Southern analysis showing clonal pattern of RadLV proviral integrations in C1-V13D progeny cell lines isolated following primary [ET4-C, ET4-D], secondary [ET4-D(T1-C), ET4-D(T1-E)] and tertiary [ET4-D(T1-E)M7A), ET4-D(T1-E)M7C] intrathymic passage. The probes used were (a) LTR, (b) p15E and (c) L. The DNA in this analysis was digested with *Pst*I. CBA/H thymus (Thy) DNA was used as the control. DNA size markers (kb) are indicated.

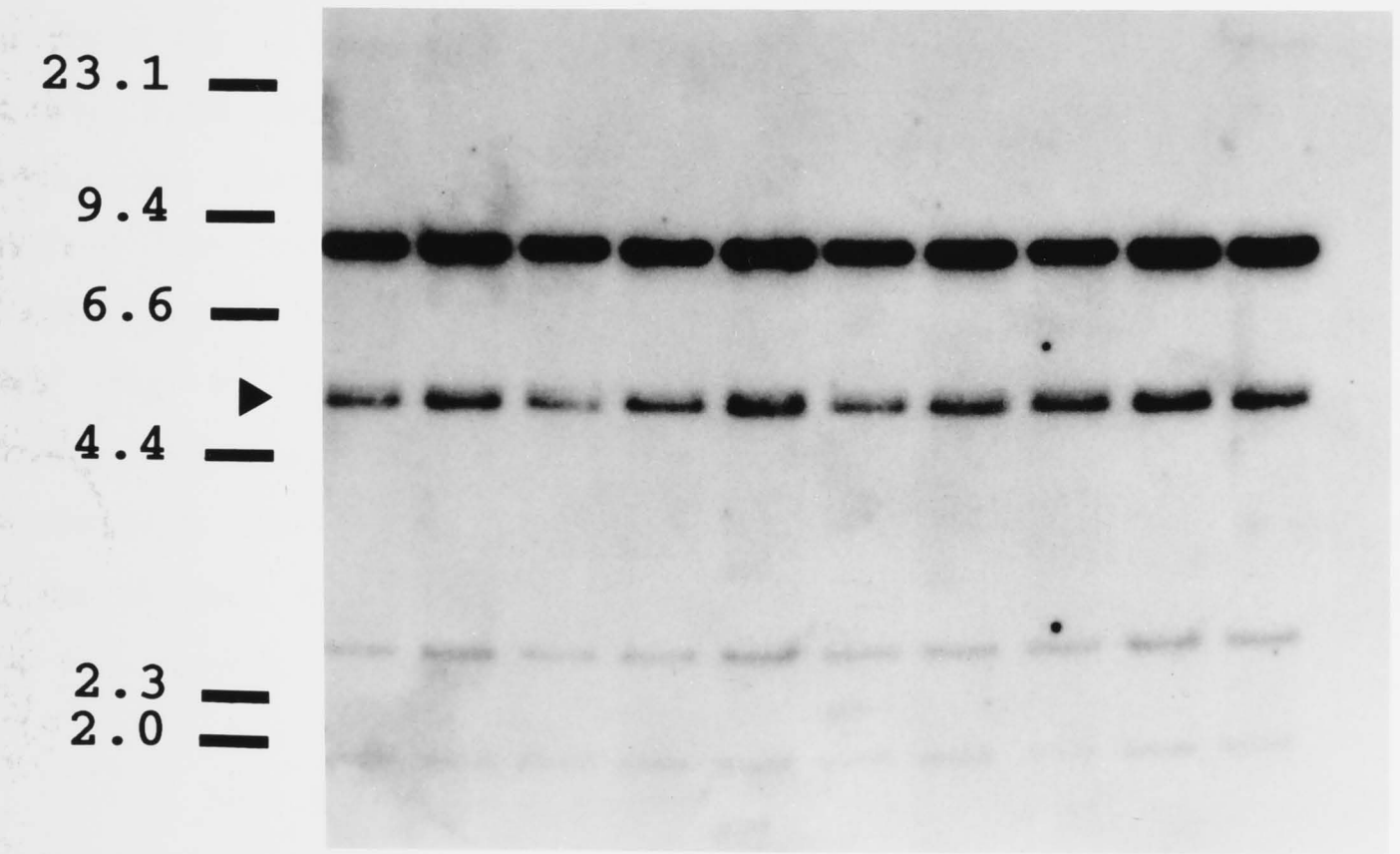
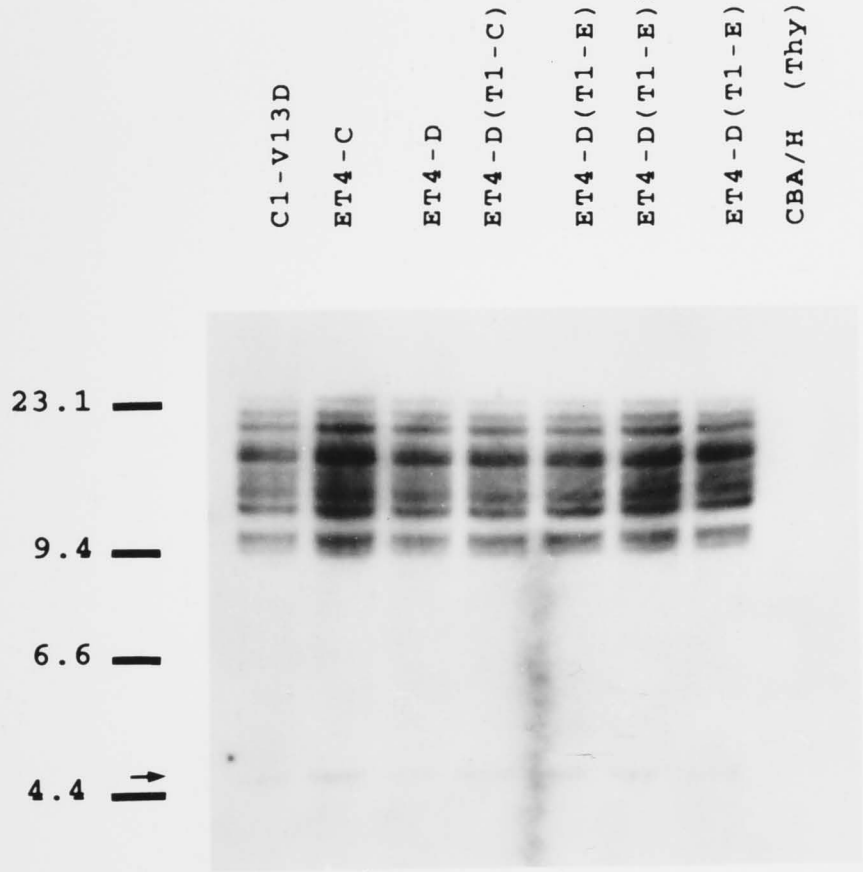


Fig. 7 Southern analysis using the gp70 *env* probe to detect changes in the pattern of RadLV integration in C1-V13D primary clones and 2 tertiary clones, ET4-D(T-E)M4C & ET4D(T1-E)M4D. DNA was digested with *Pst*I. The 5.5kb fragment (arrowed) also hybridises with the *pol*, L, LTR and p15E probes. CBA/H thymus (Thy) DNA was included as the control. DNA size markers (kb) are indicated.

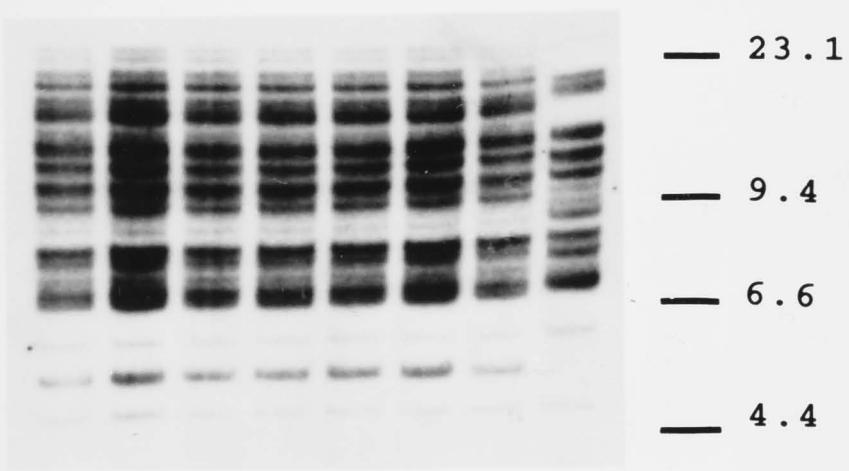
The pattern of RadLV integration was also analysed in *Eco*RI- and *Hind*III-digested DNA using the RadLV-specific gp70 *env* probe (Figs. 8a & 9, respectively, some data not shown). At least 10 fragments of size >9.4Kb were observed in *Eco*RI-digested C1-V13D DNA and in DNA isolated from clones representing primary, secondary and tertiary thymic tumours (Fig. 8a). This suggested at least 10 different viral integration sites. Since this pattern was maintained through several thymic passages, it confirmed the clonal descent of the reisolated clones and also indicated that no further integration events involving RadLV or RadLV-like viruses had occurred, which could be associated with oncogenic progression in these clones. By reprobing the same blot, it was observed that the 4.6kb band (arrowed, Fig. 8a) did not hybridise with either the L, or *pol* probes (Figs. 8b & 8c), and could represent a defective virus lacking the *pol* and *gag* genes, or a recombinant virus carrying an internal *Eco*RI site 3' to the *pol* gene. A complex but conserved band pattern was also detected using the gp70 *env* probe on *Hind*III-digested DNA from a range of primary, secondary and tertiary clones (Fig. 9). Again, this suggested multiple viral integrations, many carrying internal *Hind*III sites which presumably arose by recombination. These were present in C1-V13D, before it was passaged into the thymus. A similar pattern was also seen in other clones studied (data not shown).

New bands were observed when C1-V13D was compared with its cloned progeny using the *pol* probe. These differed only slightly in size. These new fragments were not uniformly present in all clones analysed and were detectable only in *Eco*RI- and *Pst*I-digested DNA (Figs. 8c & 10). These bands did not coincide with bands detectable with other probes. However, it was possible that the complex pattern of viral integration detected with some probes, eg. LTR, could be obscuring other faint new bands (Figs. 6a & 11). As shown in Figure 3, CBA/H mice carry no viral genes related to RadLV/V13 gp70 *env*. The new viral bands detected with the *pol* probe appeared to lack *env* sequences homologous to RadLV, indicating that they probably arose by recombination involving viruses which do not carry RadLV or RadLV-like envelope sequences present in C1-V13D or CBA/H host thymus.

a)



b)



c)

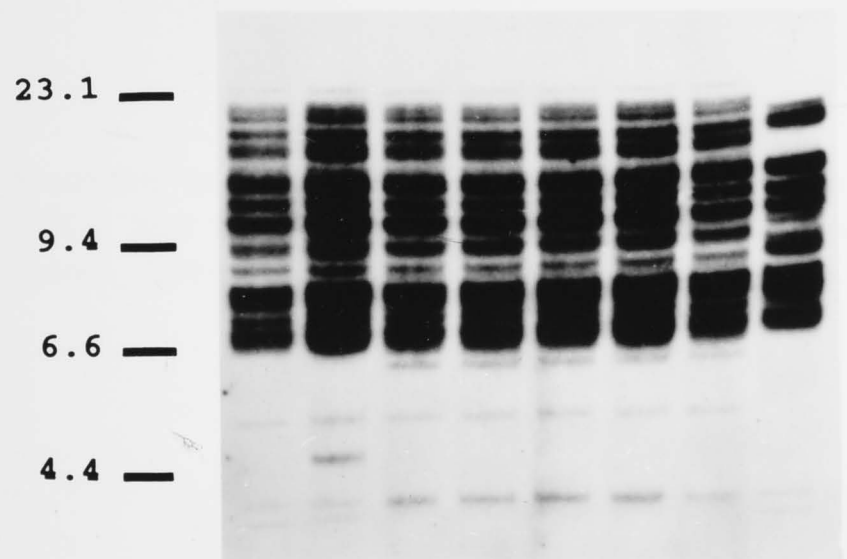


Fig. 8 Southern analysis of retroviral integration in DNA derived from 1°, 2° and 3° clones of C1-V13D (as described in Fig. 6) using *Eco*RI. Virus integration was assessed using (a) gp70 *env* (b) L and (c) *pol* probes. The 4.6kb fragment (arrowed) does not hybridise with either the L or *pol* probes. (c)*pol* changes were observed in all C1-V13D clones. CBA/H thymus (Thy) DNA was used as the control. DNA size markers (kb) are indicated.

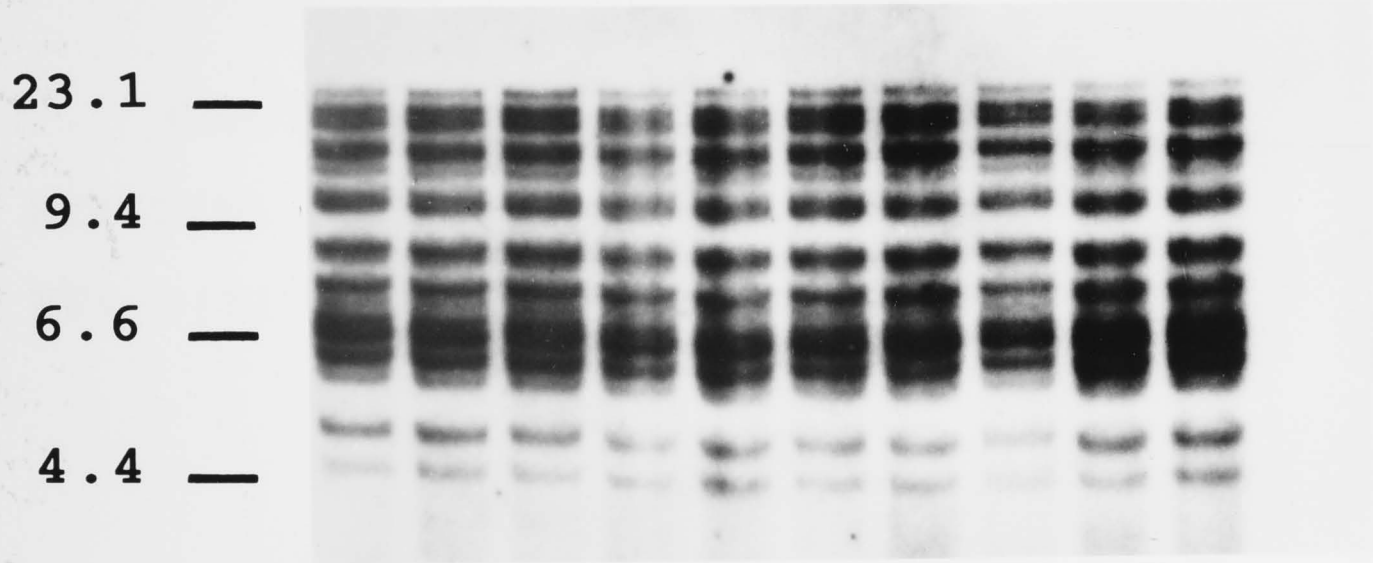
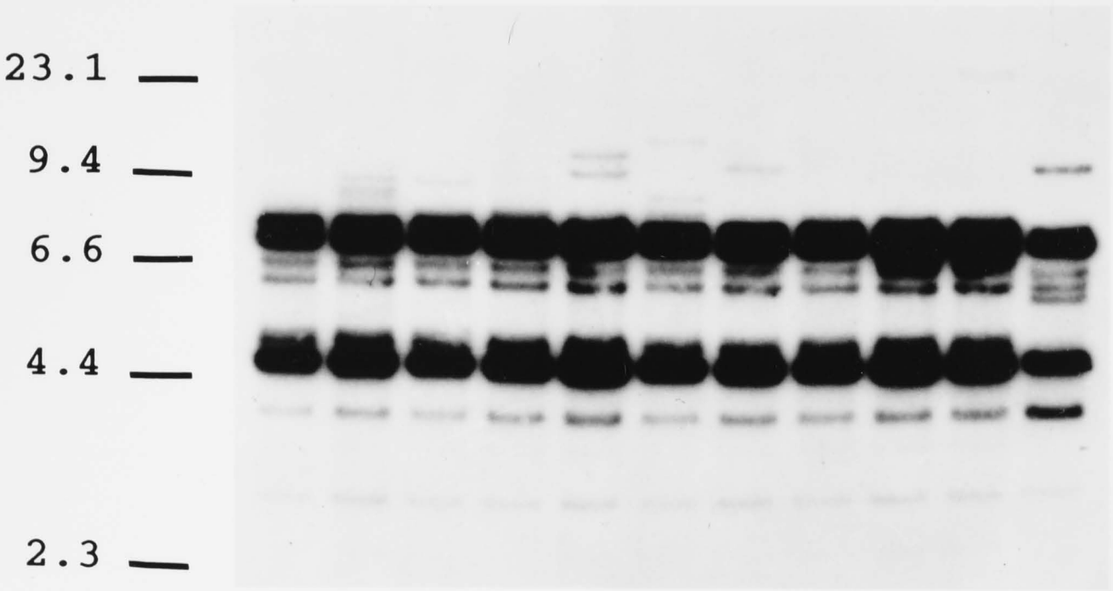


Fig. 9 Southern analysis of C1-V13D and its progeny lines using *Hind*III-digested DNA and the gp70 *env* probe. CBA/H thymus (Thy) DNA was used as the control. DNA size markers (kb) are shown.



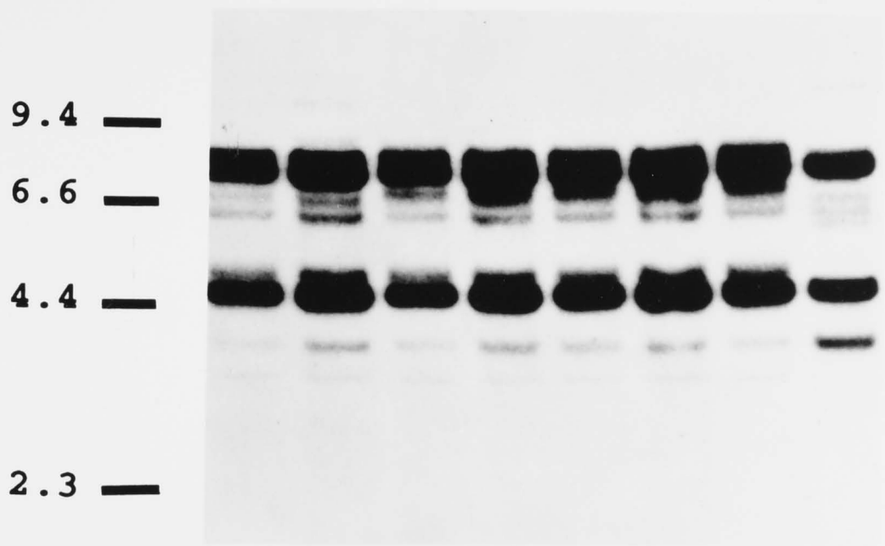


Fig. 10 Southern analysis using the *pol* probe which detected new retroviral recombination and/or integration events in randomly selected C1-V13D progeny cell lines. The DNA was digested with *Pst*I. CBA/H thymus (Thy) DNA is used as the control. DNA size markers (kb) are indicated.

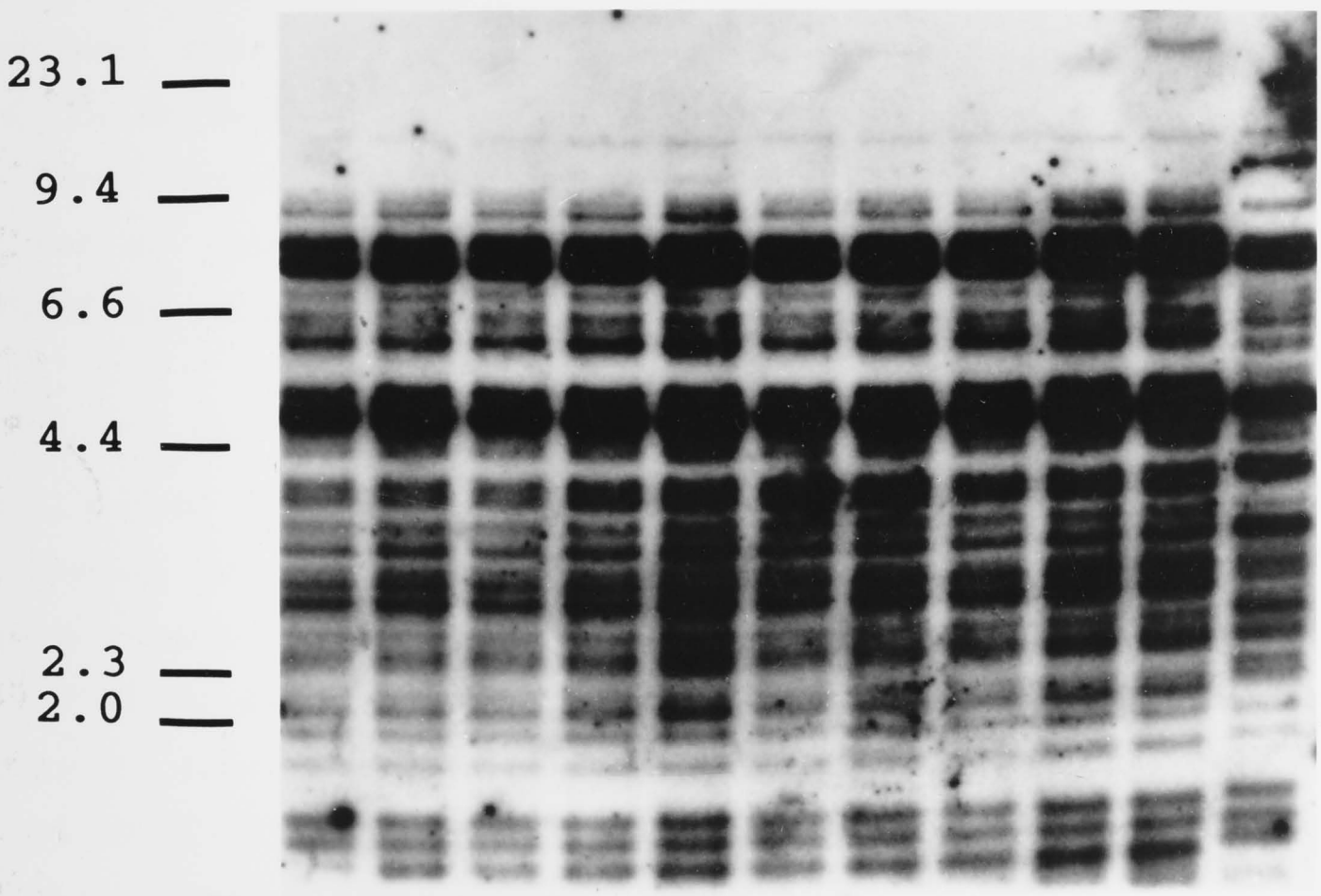


Fig. 11 Southern analysis using the LTR probe on *Pst*I-digested DNA from a number of primary and tertiary C1-V13D progeny cell lines. C1-V13D and CBA/H thymus (Thy) DNA are included as controls. DNA size markers (kb) are shown.

New fragments detectable with the *pol* probe in DNA digested with *Pst*I were larger than 8.2kb, indicating the loss of at least one *Pst*I site from the viral LTR (Fig. 10). These new recombination products differ from most full length murine retroviruses which typically contain one *Pst*I site within each LTR, giving an 8.2kb viral fragment upon *Pst*I digestion (Chattopadhyay *et al.*, 1982). New viral recombination and/or integration was apparent only in some clones isolated after primary thymic passage. The viral integration pattern in the ET4-A clone, for example, showed no change and was similar to that seen in C1-V13D (Fig. 10). Tumorigenicity studies comparing ET4-A, B, C and D clones from the primary ET4 thymic tumour (Table 1) argue against the importance of these new recombination events in oncogenic progression, since all clones were found to be equally tumorigenic in mice (Fig.10; Southern analysis of ET4-B not shown). It was concluded that these recombination events were probably incidental and random and had no significant bearing on the increased tumorigenic potential exhibited by primary clones represented in this study.

The secondary [ET4-D(TI-E)] and tertiary [ET4-D(TI-E)M7A] clones, derived from serial passage of the primary ET4-D clone through the thymus, also did not show any further change in the viral integration pattern, as indicated by the *pol* probe. This was confirmed using *Pst*I and *Eco*RI restriction analysis (Figs. 8c & 10b). Since the increasing tumorigenicity observed with successive intrathymic passage of C1-V13D could not be correlated with any further viral integration and/or recombination events, it is very probable that the changes detectable with the *pol* probe in this model are associated only with the primary growth of C1-V13D in the thymus, ie. the first passage.

4.4 Discussion

This study was aimed at determining whether further retroviral integration and recombination events were associated with the increased tumorigenic potential observed in progeny clones of C1-V13D, derived after repetitive intrathymic passage. Electron microscopy demonstrated the presence of numerous type C virus particles in C1-V13D

TABLE 1 Increased tumorigenicity of clones derived from the ET4 thymic tumour

Cell line	*Clone*	*Incidence of tumour formation*			
		ip	iv	sc	it
1º Tumour					
ET4	A	2/3	3/3	0/3	NT
ET4	B	3/3	3/3	0/3	NT
ET4	C	3/3	3/3	0/3	NT
ET4	D	1/3	3/3	0/3	11/11
Control					
C1-V13D		0/3	0/3	0/3	12/15

The ET4 thymic tumour which developed following inoculation of $5x10^5$ C1-V13D cells intrathymically into irradiated (4Gy) CBA/H mice was explanted and cultured *in vitro*. C1-V13D progeny were isolated, subcloned twice and tested for tumorigenicity in similarly irradiated CBA/H hosts by inoculation via different routes. Death occurred within 14 - 21 days post-inoculation. Mice were studied for up to 15 months.
NT: not tested

progeny cell lines, as opposed to few or none seen in the original C1-V13D cell line. A parallel situation exists in HIV infection where the thymic microenvironment has been found to upregulate expression of the virus in chronically infected cells (Schnittman *et al.*, 1991). Increased virus production and the presence of viral gene products have been correlated with oncogenesis in the case of MoMuLV-induced lymphomas (Tsichlis, 1987). In the case of avian erythroblastosis virus, continual virus production and the presence of viral products was found to be necessary for maintenance of the leukemic state of infected cells (Graf *et al.*, 1978). The increased number of virus particles seen in C1-V13D cells after intrathymic passage may function similarly, contributing to the oncogenic process.

In comparison to germline DBA/2j liver cells, C1-V13D cells carry many full length RadLV-like proviruses in their genome. This was indicated by Southern analysis on *Eco*RI-digested DNA using the ecotropic RadLV gp70 *env* probe. This probe appears to be specific for RadLV and RadLV-like envelope sequences and differs from the AKR ecotropic *env* probe used by Moore & Chan (1982). This probe was derived from λ-AKR 623, which was cloned by Lowy *et al.* (1980). With this AKR ecotropic *env* probe (Fig. 1), single fragments of size 7.0kb and 6.5kb were detected in *Hind*III-digested DNA from CBA and DBA mice, respectively. In the experiments described here using the RadLV gp70 *env* probe, no RadLV-like ecotropic envelope sequences were detected in CBA/H liver DNA, while at least two proviruses, one with an internal *Hind*III site, were detected in DBA/2j liver DNA.

It is not possible to determine how many retroviral integration events had occurred at the time of initial virus infection of the C1-V13D cell line, or to determine whether further retroviral events occurred during the long process of adaptation of the cell line to *in vitro* culture. Studies are currently in progress to investigate this process more fully. Restriction mapping of integrated viruses in C1-V13D using *Pst*I, *Eco*RI and *Hind*III has indicated that many proviruses present in C1-V13D are recombinant viruses (see Chapter 5). Many of these have at least one new restriction site in their genome and differ from the original RadLV/V13 clone which does not contain internal *Eco*RI or *Hind*III sites.

There is only the single *Pst*I site in each LTR (Rassart *et al.*, 1986). Some of the viruses in C1-V13D could be either MCF, ecotropic or xenotropic viruses. These viruses have been shown to carry internal *Hind*III and/or *Eco*RI sites (Rands *et al.*, 1981; Chattopadhyay *et al.*, 1982). Multiple retroviral integrations in C1-V13D appeared to be associated with the capacity of cells to proliferate continuously and autonomously *in vitro*. However, C1-V13D is not fully transformed and further transformation has been achieved only after replication within the thymic microenvironment.

The leukemogenic potential of many murine leukemia viruses, including recombinant viruses has been mapped to *env* and *LTR* determinants (Lenz *et al.*, 1984; Holland *et al.*, 1985; Vogt *et al.*, 1985; Rassart *et al.*, 1986 & 1988) and in a few cases, to the *gag* (Aziz *et al.*, 1989) or *gag/pol* genes (Rassart *et al.*, 1983; Lenz *et al.*, 1983; Tsichlis, 1987). New retroviral integration and/or recombination events involving the *pol* gene were detected in many clones isolated after primary intrathymic passage of C1-V13D. Of four clones derived from ET4, ie. ET4-A to D, only some showed recombination involving *pol*. However, all were shown to be tumorigenic *in vivo* (Table 1). ET4-A, with no detectable *pol* changes, was as tumorigenic as clones having new *pol* changes, indicating that this event was probably not directly related to the observed increase in tumorigenicity. On subsequent intrathymic passage of these clones, no further retroviral changes involving RadLV or other recombinant viruses were observed. This occurred despite an increase in tumorigenicity in progeny clones with each thymic passage. This confirmed that retroviral integration or recombination was not required for increased tumorigenicity which occurred with passage through the thymus. The complex retroviral integration pattern of ET4-D, detectable with the *pol* probe, was maintained in the tertiary clone ET4-D(T1-E)M7A. These results contrasted with studies in the AKR and Molony MuLV models, where viral recombination was shown to play a significant role in the oncogenic process (reviewed in Section 1.5.3). Results presented here also dissociated new retroviral integration or recombination events from differentiation events observed to occur in C1-V13D cells during thymic residence (Chapter 3). While differentiation was observed to occur consistently *in vivo* and with each intrathymic

passage (Chapter 3), the occurrence of retroviral changes involving the *pol* sequence shown in this chapter were random or incidental and bore no relationship to the progressive increase in tumorigenicity.

Data presented here cannot be taken to undermine the importance of retroviral integration and recombination events in the transformation process. However, in the case of C1-V13D, it would appear that genetic changes related to retroviral integration or recombination had already occurred during initial *in vitro* infection of C1-V13D and the long term adaptation to tissue culture. These were sufficient to confer proliferative capacity and arrest maturation of the target cell. However, as shown in Chapter 3, C1-V13D could proliferate and undergo limited differentiation if located in the correct microenvironment. In the absence of new retroviral events which can be associated with oncogenic progression in C1-V13D, the possibility that oncogenic progression is mediated by genetic changes associated with the differentiation of C1-V13D in response to the thymic microenvironment becomes more likely. This would also be consistent with data presented in Chapter 3. Any genetic changes may or may not act in concert with proto-oncogenes already functionally upregulated or dysregulated by retroviral integrations which occurred during the initial *in vitro* phase of C1-V13D immortalisation.

SUMMARY

Retroviral recombination and reintegration into the host genome are common events during retroviral replication within cells. In some cases, these events have been shown to contribute to the oncogenic process, either through the generation of more leukemogenic viruses through recombination or by dysregulation of cellular genes as a result of further proviral integration events. Southern analysis was performed to investigate retroviral integration events in C1-V13D and progeny cell lines, which were isolated after first, second and third passages intrathymically. Several different viral gene probes and restriction enzymes were used to determine whether further retroviral events were associated with the increased tumorigenicity of late passage clones. In

general, there was no evidence of reintegration of RadLV-like viruses detectable with the specific RadLV gp70 *env* probe. A large number of C1-V13D progeny lines demonstrated the presence of new recombinant viruses involving the *pol* gene. These events did not correlate with an increase in tumorigenicity noted amongst C1-V13D progeny. It is proposed that the initial retroviral integration events were sufficient to partially transform C1-V13D cells *in vitro*. While replicating in the thymic microenvironment, C1-V13D cells undergo neoplastic differentiation and further transformation towards a more tumorigenic phenotype. It is possible that genetic changes associated with differentiation within the thymus may have affected proto-oncogenes involved in cellular growth and differentiation.

CHAPTER 5

Analysis of viral and flanking genomic DNA

5.1 Introduction

Data presented in the preceding chapter indicated that multiple retroviral events had occurred during *in vitro* derivation of C1-V13D. Subsequent retroviral events which occurred during replication of C1-V13D in the thymus appeared to be unrelated to the oncogenic process. It was hypothesised that the retroviral integration and recombination events following *in vitro* infection and subsequent culture were sufficient to immortalise C1-V13D, partially transform it and simultaneously arrest its maturation. Replicating under strong selective pressure in the thymic microenvironment, C1-V13D could be induced to differentiate, indicating that while differentiation was blocked *in vitro*, it was not an irreversible event (Chapter 3).

The hypothesis that tumour cells continue to undergo neoplastic differentiation in association with oncogenic progression has been proposed in this study. This was based on evidence that some cellular proto-oncogenes involved with tumorigenesis also play an important role in regulating cellular proliferation and differentiation (reviewed in Chapter 1). The experiments described in this chapter were initiated with the objective of finding novel cellular proto-oncogenes, whose functions have been disrupted due to retroviral integration and/or recombination, leading to immortalisation, maturation arrest and partial transformation of C1-V13D. The strategy involved construction of a genomic library from C1-V13D and selection for clones carrying the RadLV gp70 *env* sequence. Viral and genomic DNA flanking the site of retroviral integration were analysed using restriction mapping, Southern analysis using specific viral probes and sequencing.

5.2 Materials and Methods

5.2.1 Genomic library construction

5.2.1A Partial digestion of C1-V13D DNA with Sau3AI

Genomic DNA was prepared as described in Section 2.2.8A. The construction of a genomic library was carried out according to Promega's Protocols and Application Guide (Titus, 1991). Partial digestion of C1-V13D DNA with *Sau*3AI was carried out to generate overlapping DNA fragments of 15-23kb. The digestion was optimized on a small scale using various dilutions of the enzyme. After 30 minutes incubation at 37°C, the digestions were stopped by the addition of 0.5M EDTA/pH 8.0 and sample buffer (38% sucrose, 0.1% bromophenol blue, 67mM EDTA). The degree of digestion was visualized on a 0.65% agarose gel. The amount of enzyme needed to produce the desired 15-23kb fragments could be determined from the intensity of fluorescence, which was directly related to the mass distribution of DNA. To minimise the number of DNA molecules smaller than the desired size in the large scale preparation, half the amount of enzyme that produced the maximum amount of fluorescence in the desired size range was used.

DNA (100µg) was digested in a large scale preparation using conditions identical to that used in small scale reactions. The digested DNA was extracted with an equal volume of TE-saturated phenol:chloroform (24:1) by gentle inversion and subsequent centrifugation at 15,800 x *g* in a microfuge for 5 minutes. The upper aqueous phase was transferred to a fresh tube and extracted once with an equal volume of chloroform:isoamyl alcohol (24:1). After centrifuging again to separate the phases, the upper aqueous phase was removed into a fresh tube. The DNA was precipitated at -20°C for 30 minutes by addition of 0.1X volume of 7.5M ammonium acetate and 2X volumes of ethanol, pelleted again at 15,800 x *g* for 10 minutes and rinsed twice with 70% ethanol. It was dried under vacuum and resuspended in 500µl TE buffer/pH 7.4.

The DNA was size fractionated on a 10-40% continuous glycerol gradient for 24 hours at 83,000 x *g* (18°C) in a SW41Ti rotor (Beckman, USA). Fractions (about 5 drops per fraction) were collected by puncturing a hole at the bottom of the tube. A small aliquot was removed from each fraction and electrophoresed on a 0.7% agarose gel to check the size distribution of the digested products. Fractions containing DNA of the desired size were pooled and precipitated using 7.5M ammonium acetate and ethanol. The DNA was pelleted and dried as described previously, resuspended in an appropriate amount of TE buffer and stored at -20°C.

5.2.1B Ligation to λGEM-11 BamHI arms and preparation of host strains for infection

The vector used for library construction was λGEM-11 (Promega, USA). For optimal yield of recombinant DNA molecules appropriate for packaging into λ particles, the best molar ratio of λ arms (43kb) to genomic insert (averaging 15-23kb) was tested and established to be 1:1. The arms and insert were added together and precipitated overnight at -20°C with 7.5M ammonium acetate/pH 7.0 and 2X volumes ethanol. The pellet obtained after centrifugation was washed twice with 70% ethanol and vacuum dried. Ligation was carried out for 16 hours at 15°C in a water bath in 1X ligase buffer (Promega, USA) containing 1 Weiss unit of T4 DNA ligase per µg of DNA. On the same day, a colony of KW251 cells (*sup*E44, *sup*F58, *gal*K2, *gal*T22, *met*B1, *hsd*R2, *mcr*B1, *mcr*A-, *arg*A81:Tn10, *rec*D1014, F^-) [Promega, USA] was inoculated into 3ml LM medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 10mM $MgSO_4$) containing 0.2% maltose. The cells were grown overnight at 37°C with vigorous agitation. Fifty microlitres of the overnight culture was inoculated into 50ml of LM medium containing 0.2% maltose. The cells were grown at 37°C with vigorous agitation until the absorbance at OD_{600} reached 0.5-0.6. The cells were then pelleted, resuspended in half the volume of ice-cold 10mM $MgSO_4$ and kept on ice, ready for infection with recombinant phage particles.

5.2.1C *In vitro packaging and infection of KW251 cells*

In vitro packaging of recombinant phage DNA was carried out using the Gigapack Gold packaging extract purchased from Stratagene, USA. The phage was packaged according to the supplier's recommendations. Briefly, DNA was added immediately to thawing freeze-thaw extracts containing phage packaging proteins, mixed quickly and placed on ice. Sonic extracts (15µl) containing phage preheads were then added. Phage proteins, preheads and DNA were mixed well without introduction of air bubbles, centrifuged quickly in a microfuge (15,800 x *g*, 3-5 seconds) and incubated at 22°C for 2 hours. A 500µl volume of phage dilution buffer (SM) containing 0.02M Tris/pH 7.4, 0.1M NaCl and 0.01M $MgSO_4$ was then added. Packaged phage particles were incubated with KW251 cells at 37°C for 20 minutes, mixed with the appropriate amount of top agar (0.7% Sigma Type II agarose in LM) at 45°C and plated immediately onto 15cm LB agar plates prewarmed to 37°C. To construct a library that was representative of the whole genome, 30 platings were made on 15cm plates to achieve a total of $8.4x10^5$ clones for screening. The plates were incubated overnight at 37°C for 8 hours.

5.2.2 *Screening of recombinant λ clones carrying the RadLV gp70 env sequence*

Plates were cooled at 4°C for approximately 2 hours to harden the top agar layer before plaque lifts were made. A 15cm diameter sterile Hybond N^+ membrane (Amersham, Australia) was laid on top of each agar plate for 1 minute, removed, and the DNA on the membrane was fixed by placing it for 20 minutes (with the side in contact with the agar facing up) on a piece of Whatman No. 3 filter paper pre-soaked with 0.4M NaOH. After fixation, the membranes were washed at least thrice with 2XSSC. Specific nucleic acid sequences were detected as described previously (Chapter 2, Section 2.2.8E). RadLV gp70 *env* positive recombinant phages were harvested with a sterile glass pasteur pipette by slicing off the top agarose layer and placing it in 1ml phage storage buffer, PSB (0.02M Tris/pH 7.4, 0.1M NaCl, 10mM $MgSO_4$, 2% gelatin) containing 30µl chloroform. The samples were placed on a

rotator at 22°C for at least 2 hours or at 4°C overnight to allow the phage particles to diffuse out of the agar into the medium.

Selected clones were replated twice and plaque lifts were taken after each replating. The purity of the clones was determined by hybridization to ^{32}P-labelled RadLV gp70 *env* probe.

5.2.3 Preparation of λ DNA from phage lysates

A volume of 2µl purified phage particles in PSB from each individual λ clone was spotted onto freshly plated KW251 cells and incubated at 37°C overnight. A plaque of size 5mm in diameter usually yielded sufficient infectious phage particles for preparation of a good phage lysate. The 5mm plaques were placed in 200µl SM buffer with a drop of chloroform. The phage particles were allowed to diffuse out at room temperature for at least 2 hours, centrifuged to remove agar and chloroform and the supernatant, containing phage particles, was used to infect 500µl of an overnight culture of KW251 cells which had been resuspended in 10mM $MgSO_4$. The phage/cell mixture was incubated at 37°C for 20 minutes to facilitate the adsorption of phage particles onto cells. Prewarmed LB medium (20ml) was then added and the infection was allowed to continue with vigorous agitation at 350rpm until complete lysis of bacteria was achieved. Sodium chloride was added to achieve a final concentration of 1M and the mixture was left on ice for 45 minutes to allow phage particles to dissociate from the cell debris. As an optional step, the lysed mixture could be incubated with 100mg/ml of DNaseI and 100mg/ml of boiled RNase A for a further 45 minutes at 37°C before the addition of NaCl. The cell debris was removed by centrifugation at 2000 x *g* for 20 minutes, polyethylene glycol (PEG) 6000 was added to a final concentration of 10% and the mixture was kept at 4°C overnight. After centrifugation for 10 minutes at 17,200 x *g* in a Sorvall centrifuge using a SS34 rotor (Du Pont, Australia), the phage/PEG pellet was resuspended in SM buffer and extracted with an equal volume of chloroform. The aqueous phase was transferred to an ultracentrifuge tube and the phage particles pelleted at 110,000 x *g* in a SW55 Ti rotor for 2 hours at 4°C. The pellet was dissolved slowly in 400µl SM buffer at 4°C overnight. The phage particles were then placed in a phage caesium

chloride (CsCl) gradient (2% sodium N-lauroyl sarcosine, 0.005M Tris-HCl/pH 8.0, 0.04M EDTA/pH 8.0, 400µg/ml ethidium bromide, 63% w/v CsCl) according to Buckley & Goding (1988) and ultracentrifuged at 348,000 x *g* overnight using a TLA100.4 rotor (Beckman, USA). The phage DNA band was extracted with a 21G needle attached to a 1ml syringe and the ethidium bromide was removed by extracting thrice with an equal volume of water saturated butanol. The phage DNA was precipitated with an equal volume of isopropanol at -20°C, washed twice with ethanol, vacuum dried and resuspended in an appropriate amount of dd$H_2$0 or TE/pH 7.4. The DNA was stored at -70°C.

5.2.4 Strategy for mapping λ clones carrying viral and flanking genomic DNA

5.2.4A Southern analysis of endonuclease restricted λ-DNA

Composite physical maps were deduced from partial restriction endonuclease mapping and sequence analysis of DNA regions in the immediate vicinity of λ arms using the T7 (5' TAA TAC GAC TCA CTA TAG G) and SP6 (5' ATT TAG GTG ACA CTA TAG) sequencing primers (Fig. 1). Four restriction enzymes were used, namely, *Sfi*I, *Hind*III, *Eco*R I and *Pst*I. The use of *Sfi*I, which is present in the λGEM-11 vector at both ends of the polycloning site allowed the size of most inserts to be determined since the 8-base *Sfi*I recognition sequence occurs infrequently in genomic DNA. The *Hind*III site occurred only once in the 9kb right arm of λGEM-11 and was therefore useful for positioning of the insert with respect to the vector. Although there are numerous *Pst*I restriction sites in the left 20kb arm of λGEM-11, none was present in the 9kb right arm. Since *Pst*I typically cuts at each LTR (Chattopadhyay *et al.*, 1982), this enzyme could therefore be used to size the 3' LTR/host junction using the LTR probe. *Eco*RI sites are not present in either the vector or the RadLV provirus and could provide useful information on the size of the genomic DNA flanking the LTRs and also indicate new sites which may have been generated by recombination within the virus. The restricted λ clones were electrophoresed through agarose gels, transferred to Hybond N^+ membranes and hybridised to RadLV proviral DNA probes as described in Section 2.2.8 for Southern analysis.

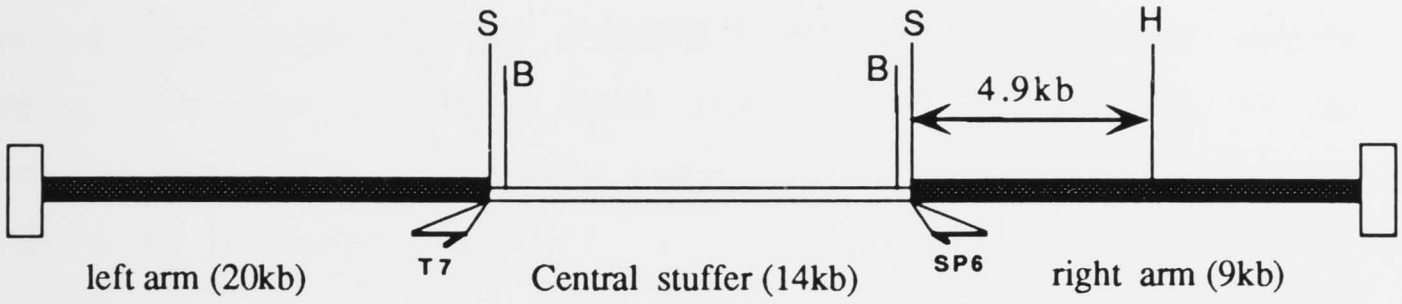


Fig. 1 A simplified structural map of the λGEM-11 vector used for C1-V13D genomic library construction (not drawn to scale). The vector was purchased as dephosphorylated *Bam*HI (B) arms (Promega, USA). The *Sfi*I (S) sites at the ends were used to determine the size of the cloned inserts while the *Hind*III (H) site was instrumental in the positioning of the provirus and its flanking genomic sequences. The SP6 and T7 RNA polymerase transcription start sites are indicated.

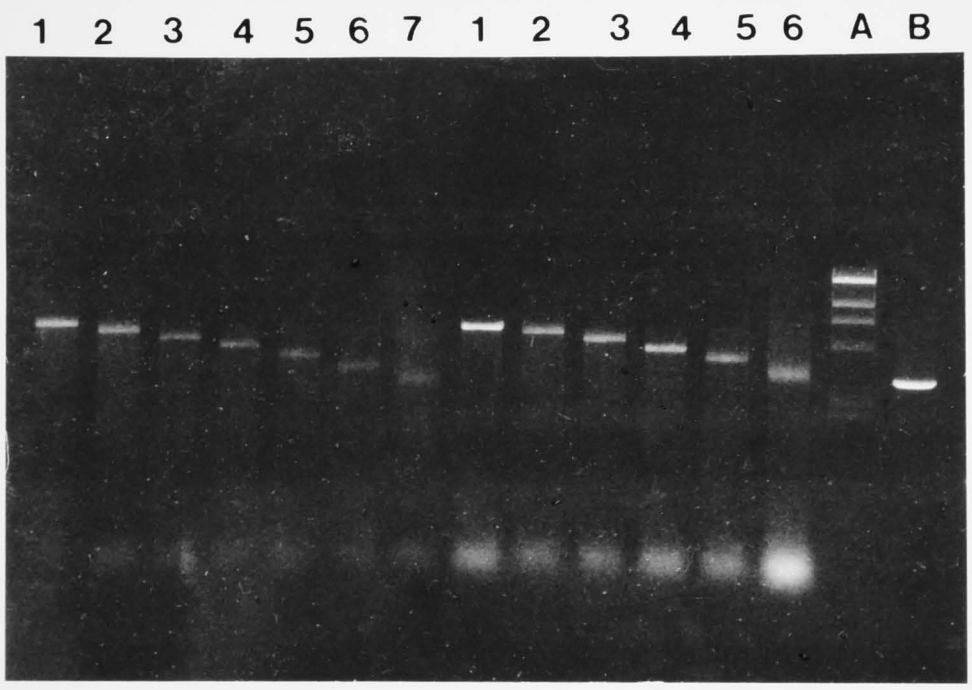


Fig. 2 An ethidium bromide stained gel illustrating unidirectional deletion using Exonuclease III (*Exo*III). This method allowed the rapid construction of plasmids containing progressive unidirectional deletions of any inserted DNA. This facilitated efficient sequencing of large DNA fragments. *Exo*III deletions of two plasmid clones are shown here. Numbers represent time points at one minute intervals. Lane (A) represents *Hind*III-digested DNA (kb) and lane (B) represents the 3kb linearised pGEM7Zf(+) plasmid.

The position of viral probes are shown in Figure 1 (Chapter 4). Subcloning and sequencing of *Hind*III and *Pst*I fragments of interest (including host-virus junctions) were also carried out as part of the mapping strategy. Procedures for subcloning and transformation have been described in Section 4.2.4.

5.2.4B Isolation and subcloning of LTR-host junction DNA

*Hind*III-fragments comprising 5' LTR-host junction sequences from λ-1 (~3.2kb), λ-7 (~2.8kb) and λ-17 (~4.8kb), and the 3' LTR-host junction from λ-1 (~8.0kb) were subcloned into the plasmid pGEM7Zf(+) at the *Hind*III polycloning site according to the procedure described in Section 4.2.4. A *Hind*III LTR-host junction fragment of λ-27 (~1.5kb) was also subcloned. Fragments subcloned into plasmids were given the prefix pλ. They were therefore designated as pλ-1f2(H), pλ-1f1(H), pλ-7G, pλ-17f2(D) and λ-27f(C)1, according to the λ clone from which they were derived. Plasmid DNA was prepared as described in Section 4.2.4E.

5.2.4C Cycle sequencing with dye primers

Double stranded λ DNA (7μg) or plasmid DNA (1μg) for sequencing was denatured in 0.25M NaOH/EDTA for 5 minutes at 37°C, placed on ice and neutralised with 3M sodium acetate/pH 6.0 to give a final concentration of 1M. The denatured DNA was precipitated with the addition of 3X volumes 95% ethanol, kept at -70°C for 15 minutes or -20°C overnight. The pellet was washed, vacuum dried and resuspended in an appropriate amount of water. Cycle sequencing was performed in the FTS-1 thermal cycler (Corbett Research, Australia) in 4 sealed capillary tubes each containing 20μl cocktail of d/ddNTP mix, 0.4pmol/μl dye primer (T7 or SP6), 1X sequencing buffer, 0.6U AmpliTaq and an appropriate amount of DNA template. All reagents were supplied in the Taq Dye Primer Sequencing kit T7/SP6 (Applied Biosystems Inc., California, USA). Cycling parameters were 2 minutes at 94°C for 1 cycle, followed by 30 cycles of 94°C for 20 seconds, 53°C for 20 seconds and 72°C for 60 seconds. The 4 reactions from one sample were pooled at the end of the sequencing reaction, precipitated with 1.5μl of 3M sodium acetate/pH

5.2 and 80µl of 95% ethanol, pelleted, washed, and vacuum dried. The samples were electrophoresed on a 6% polyacrylamide gel containing 8% urea at 1600V for 14 hours in an automated DNA sequencer (ABI Model 373A) and analysed using the ABI Data Analysis Program software, Version 1.2.0 (1989). Electrophoresis was carried out at the Biomolecular Resource Facility in the John Curtin School of Medical Research, Canberra, Australia.

5.2.5 Analysis of flanking genomic DNA distal to the LTR

5.2.5A Identification of transcribed regions using [$\alpha^{32}P$]-dCTP labelled cDNA

^{32}P-labelled cDNA was prepared from C1-V13D polyadenylated RNA. This was used as a probe to identify transcribed regions in isolated recombinant λ-clones by Southern analysis. Total RNA was prepared as described in Section 3.2.5A and enriched for polyadenylated RNA using chromatography on oligo(dT)-cellulose as described by Sambrook *et al.* (1989). Briefly, the separation column was prepared by filling a 1ml silanized glass syringe with 0.5g of oligo(dT)-cellulose (Sigma, USA) resuspended in 0.1M NaOH. The glass syringe was plugged with silanized glass wool. A maximum of 10mg total RNA could be loaded per column. Equilibration of the column was carried out with poly A buffer (50mM sodium citrate, 0.5M NaCl, 1mM EDTA, 0.1% sodium N-lauroyl sarcosine/pH 7.5) until the pH was <8.0. Five to ten milligrams of total RNA was heated to 65°C for 5-10 minutes, cooled rapidly to room temperature and an equal volume of 2X poly A buffer was added. The RNA solution was loaded onto the column and the eluate collected into a sterile tube, heated again to 65°C for 5 minutes, re-applied to the column and collected. This process was repeated thrice. The column was then washed with 5-10 column volumes of poly A buffer or until OD_{260} absorbing material no longer eluted. Polyadenylated RNA was then eluted with 2-3 column volumes of freshly prepared and autoclaved elution buffer (10mM Tris-Cl/pH 7.6, 1mM EDTA, 0.05% SDS). Fractions of 400µl were collected into silanized Eppendorf tubes and those containing RNA (checked at OD_{260}) were pooled. The RNA was precipitated with 0.1X

volume of 3M sodium acetate/pH 5.2 and 2X volumes ethanol at -20°C overnight. It was pelleted, washed twice with 70% ethanol, vacuum dried and resuspended in a small volume of DEPC-water and kept at -70°C.

The synthesis of ^{32}P-labelled cDNA was carried out according to Sambrook *et al.* (1989). One microgram RNA was mixed with 250ng $(dN)_6$ hexamers (Pharmacia, USA) at 65°C for 10 minutes. The mixture was chilled on ice and a cocktail consisting of reverse transcriptase (RT) buffer (Promega, USA), 500-600mM each of dGTP, dTTP and dATP (Pharmacia, USA), 10mM DTT, 50U/ml RNAsin (Promega, USA) and 1000U/ml Moloney murine leukemia virus reverse transcriptase (Gibco, BRL, USA) was added followed by approximately 100µCi of [α^{32}P]-dCTP (Amersham, Australia). Synthesis was carried out at 42°C for 1 hour. The reaction was chased with 600µM cold dCTP for 15 minutes at 42°C and finally terminated with 0.5M EDTA/pH 8.0 (0.03M final concentration). RNase A (0.5µg) was added and the mixture was left at 37°C for 1 hour, extracted once with phenol and passed through a G-50 Sephadex column to remove unincorporated label, as described in Section 2.2.8D.

To check the integrity of synthesised cDNA, the labelled product was electrophoresed through a 1% alkaline agarose gel containing 0.05M NaOH and 0.001M EDTA. Loading buffer (0.3M NaOH, 6mM EDTA, 1% Ficoll, 0.15% bromocresol green, 0.25% xylene cyanol) was added to the sample before loading. Electrophoresis was carried out at 0.25V/cm with freshly prepared 50mM NaOH and 1mM EDTA as running buffer. After electrophoresis, the gel was soaked in 7% TCA (trichloroacetic acid) for 30 minutes at room temperature. It was dried overnight with paper towels and exposed to X-ray film (Kodak, Australia).

Southern blots of *Hind*III- or *Pst*I-digested λ DNA were analysed using the ^{32}P-labelled cDNA probe. All fragments hybridising to the cDNA probe but not with labelled wild-type λ or RadLV sequences were taken to represent transcribed or cDNA$^+$ genomic sequences. Some of these were subcloned into pGEM^R vectors (Promega, USA) and sequenced.

5.2.5B Isolation and subcloning of transcribed or cDNA$^+$ fragments

*Hind*III fragments were used for subcloning since this enzyme gave very clear restriction patterns and there is only one *Hind*III site present in the 9kb right arm of λGEM-11 (Fig. 1). Reprobings with ^{32}P-labelled cDNA were carried out to ensure that the right fragments were subcloned. The isolation of DNA fragments and subcloning are described in Section 4.2.4.

5.2.5C Unidirectional deletions using Exonuclease III (ExoIII)

The rapid construction of recombinant plasmids containing progressive unidirectional deletions of a DNA insert was carried out as described in Promega's Protocols and Applications Guide (Titus, 1991). This method allowed for efficient sequencing of cloned DNA fragments. Closed circular plasmid DNA was prepared as described in Sections 4.2.2D. The plasmid (5-10μg) containing the insert was doubly digested to completion using two different restriction enzymes, one generating a 3' protrusion which protected the sequencing primer binding site in the vector from *Exo*III digestion, and the other which left a 5' overhang or blunt end adjacent to the insert, from which deletions proceeded. Reaction volumes were scaled according to the amount of starting DNA and the desired number of time points. Prior to exonuclease digestion, the doubly digested DNA was extracted once with TE-saturated phenol and precipitated with 0.1X volume 2M NaCl and 2X volumes ethanol at -70°C for 15 minutes. It was then pelleted and washed once with 70% ethanol, dried under vacuum and dissolved in an appropriate amount of *Exo*III buffer (66mM Tris-HCl/pH 8.0, 66mM $MgCl_2$). The DNA was warmed to 37°C, exonuclease III [Promega, USA] (300-500U for a 6.5kb insert) was rapidly mixed in and the reaction was incubated at 37°C. At 30 second or 1 minute intervals, 2.5μl of the sample was removed into a tube containing 7.5μl freshly prepared S1 nuclease mix which had been chilled on ice (0.04M potassium acetate/pH 4.6, 0.34M NaCl, 1.35mM $ZnSO_4$, 50% glycerol, 12U S1 nuclease [Promega, USA]). When all time points had been taken, the tubes were removed from ice and kept at room temperature for 30 minutes to enable S1 nuclease digestion of the single stranded DNA overhangs. One microlitre of S1 stop buffer (0.3M Tris base, 0.05M EDTA)

was then added and the tubes were heated at 70°C for 10 minutes to inactivate S1 nuclease. Two microlitres from each reaction was removed and electrophoresed on a 0.8% agarose gel to confirm controlled digestion. Two representative *Exo*III digestions are shown in Fig. 2. The rest of the sample from each time point was transferred to 37°C and 1µl of T4 DNA polymerase mix (0.01M Tris-Cl/pH 8.0, 5mM $MgCl_2$, 2.5mM DTT, 0.5% BSA, 0.2U T4 DNA polymerase) was added to each sample. The reaction was incubated at 37°C for 3 minutes, after which 1µl of dNTP mix (0.125mM each of dATP, dCTP, dGTP and dTTP) was added. Incubation was carried out for a further 5 minutes at 37°C to ensure blunting of DNA ends, cooled to room temperature and 40µl of T4 ligase mix (0.5M Tris/pH 7.6, 10mM $MgCl_2$, 6.25% PEG, 10mM DTT, 0.25mM ATP) containing 1 Weiss unit T4 DNA ligase was added to each sample. Self ligations to repair the plasmids were carried out at 12°C overnight in a water bath. For transformation, the DNA was first precipitated, washed with 70% ethanol, vacuum dried and resuspended in an appropriate amount of water. Transformation was carried out using the heat shock method as described in Section 4.2.4B.

5.2.6 Computational analysis

Computational analysis was carried out using a variety of software packages. The FastA utility was used to fast match sequences with the GENBANK and EMBL databases. The BLAST software, a very fast sequence comparison tool developed by the National Centre for Biotechnology Information (California, USA) was also used. Both FastA and BLAST are accessible through the GENBANK ON-LINE SERVICE (GOS) and are available on the Australian National Genomic Information Service (ANGIS) network. ANGIS is operated by the Australian Genomic Information Centre, The University of Sydney.

5.3 Results

5.3.1 Partial restriction mapping of λ clones carrying the RadLV gp70 env sequence confirmed the presence of recombinant and defective viruses in C1-V13D

Phage plaques were screened using the RadLV gp70 *env* probe. A total of 30 λ clones hybridised to this probe on the first plating. They were isolated, replated and screened twice using the same probe. Ten were lost in the third plating, leaving 20 for analysis. The results are presented in Table 1. Of these, 8 clones appeared to possess the full complement of retroviral genes, ie. *gag, pol, env*, and yielded two fragments which hybridised to the LTR probe when digested with *Pst*I, representing the 5' and 3' LTR-host junctions (Table 2).

λ clones lacking viral genes, for example one or both *LTR*s, *LTR/gag* or *LTR/gag/pol* (λ- 4, 6, 7, 8, 10, 12, 15, 16, 20, and 21) may have been truncated during library construction with *Sau*3AI, which is a frequent four-base cutter. However, λ-18 and λ-26 (with 2 LTRs) have part or all of their *pol* or *gag* genes deleted at regions covered by the respective probes used. They could represent defective viruses with internal deletions in their genome.

With the exception of λ-5 and λ-11, all cloned full length viruses were found to carry an internal *Hind*III site at the *pol* or *gag* genes, as indicated by Southern analysis using the L and *pol* probes (Table 2). The presence of this restriction site distinguished them from the RadLV/V13 clone (Rassart *et al.*, 1986), which was used to generate the C1-V13D cell line (O'Neill, 1992). They appear to represent recombinants in the *gag* or *pol* region, while still retaining the RadLV gp70 *env* sequence. This concurred with Southern analysis of genomic DNA from C1-V13D using the same enzyme and the RadLV gp70 *env* probe, which had earlier indicated the presence of many proviruses having an internal *Hind*III site (Section 4.3.3, Fig. 9). *Pst*I digestion of these viruses each gave a typical 8.2kb viral fragment which hybridised to all 5 viral probes, and another fragment of variable size hybridising

TABLE 1 **Summary of Southern analysis on recombinant λGEM-11 clones using *Hind*III and *Pst*I restriction endonucleases and 5 viral probes, namely LTR, leader/*gag* (L), *pol*, gp70 *env* and p15E.**

Restriction enzymes	*Hind*III	*Pst*I	*Hind*III or *Pst*I			
Clone	*LTR*	*LTR*	*L*	*pol*	*gp70*	*p15E*
1	++	++	+	+	+	+
3	++	++	+	+	+	+
4	+	+	-	-	+	+
5	+	++	+	+	+	+
6	-	-	-	+	+	+
7	+	+	+	+	+	+
8	+	+	-	-	+	+
10	+	+	-	-	+	+
11	+	++	+	+	+	+
12	-	+	-	-	+	+
13	+++	++	+	+	+	+
15	-	-	-	-	+	+
16	+	+	-	-	+	+
17	++	++	+	+	+	+
18	+	+	+	-	+	+
19	++	++	+	+	+	+
20	-	-	-	-	+	+
21	-	-	+	+	+	+
26	+	++	-	+	+	+
27	+++	++	+	+	+	+

+, ++, +++, - represent one, two, three or no fragments detected by Southern analysis.

TABLE 2 **Southern analysis of *Hind*III- and *Pst*I-digested-λ clones using 5 viral probes, namely LTR, leader/*gag* (L), *pol*, gp70 *env* and p15E. Only λ clones carrying the full length virus are shown. Data points represent fragment sizes in kilobases.**

Clone	*Hind*III				
	LTR	*L*	*pol*	*gp70*	*p15E*
1	8.0/3.0	3.0	8.0	8.0	8.0
3	10.5/6.6	10.5	6.6	6.6	6.6
5	7.8	7.8	7.8	7.8	7.8
11	7.8	7.8	7.8	7.8	7.8
13	6.0/5.0/3.0	5.0	5.0	6.0	6.0
17	23.0/4.8	4.8	23.0	23.0	23.0
19	8.0/6.0	8.0	6.0	6.0	6.0
27	7.0/3.0/1.5	3.0	7.0	7.0	7.0
Clone	*Pst*I				
	LTR	*L*	*pol*	*gp70*	*p15E*
1	8.2/2.0	8.2	8.2	8.2	8.2
3	8.2/4.2	8.2	8.2	8.2	8.2
5	5.5/2.6	5.5	5.5	5.5	5.5
11	5.5/2.5	5.5	5.5	5.5	5.5
13	8.2/0.7	8.2	8.2	8.2	8.2
17	8.2/3.6	8.2	8.2	8.2	8.2
19	8.2/6.9	8.2	8.2	8.2	8.2
27	8.2/1.0	8.2	8.2	8.2	8.2

with the LTR probe, indicating that they are normal full length viruses (Chattopadhyay *et al.*, 1982) [Table 2].

The two clones, λ-5 and λ-11 appeared to be identical and gave two LTR fragments when digested with *Pst*I, one approximately 2.5kb and the other approximately 5.5kb. This does not appear to be a cloning artefact as a similar 5.5kb fragment was also observed in *Pst*I-digested C1-V13D genomic DNA analysed with the RadLV gp70 *env* probe (Chapter 4, Fig. 7). This 5.5kb fragment hybridised to all 5 viral probes, suggesting that it could represent a defective virus with internal deletions of some viral sequences and having a *Pst*I site either 3' to the p15E or 5' to the L sequences.

Three LTR fragments were observed by Southern analysis when λ-13 and λ-27 were digested with *Hind*III (Fig. 3). Three possibilities could account for this: 1) this represents an *in vitro* cloning artefact, 2) there is a genuine LTR structure in close vicinity of the full length virus *in situ*, or 3) recombination between two *different* proviruses at the LTR, or a single point mutation in one LTR has generated a virus with non-identical LTRs, one of which possesses a *Hind*III site. The presence of a *Hind*III restriction site at *each* LTR is not indicated since only 3, and not 4, LTR fragments were observed (Fig. 3). The presence of a third LTR (possibility 2) was proposed since LTR structures, containing no other viral genes, have been reported in endogenous chicken proviruses (Hughes *et al.*, 1981) and in MoMuLV-induced rat thymomas (Lazo & Tsichlis, 1988). It was proposed that during recombination between the ends of normal proviruses, the remainder of the viral genome would form a free circle. Under these circumstances, the rest of the viral genome could be lost from the germline, leaving behind the single copy of LTR in the host genome.

Only 2 LTR fragments were observed in both λ-13 and λ-27 after digestion with *Pst*I, one of which represents the full length viral fragment of ~8.2kb containing the 5'LTR and the other the smaller 3'LTR/host junction fragment. Because of this discrepancy, the 1.5kb *Hind*III LTR fragment from λ-27 [(pλ-27f(C)1] was subcloned and sequenced to determine the positioning of the LTRs, and to analyse the

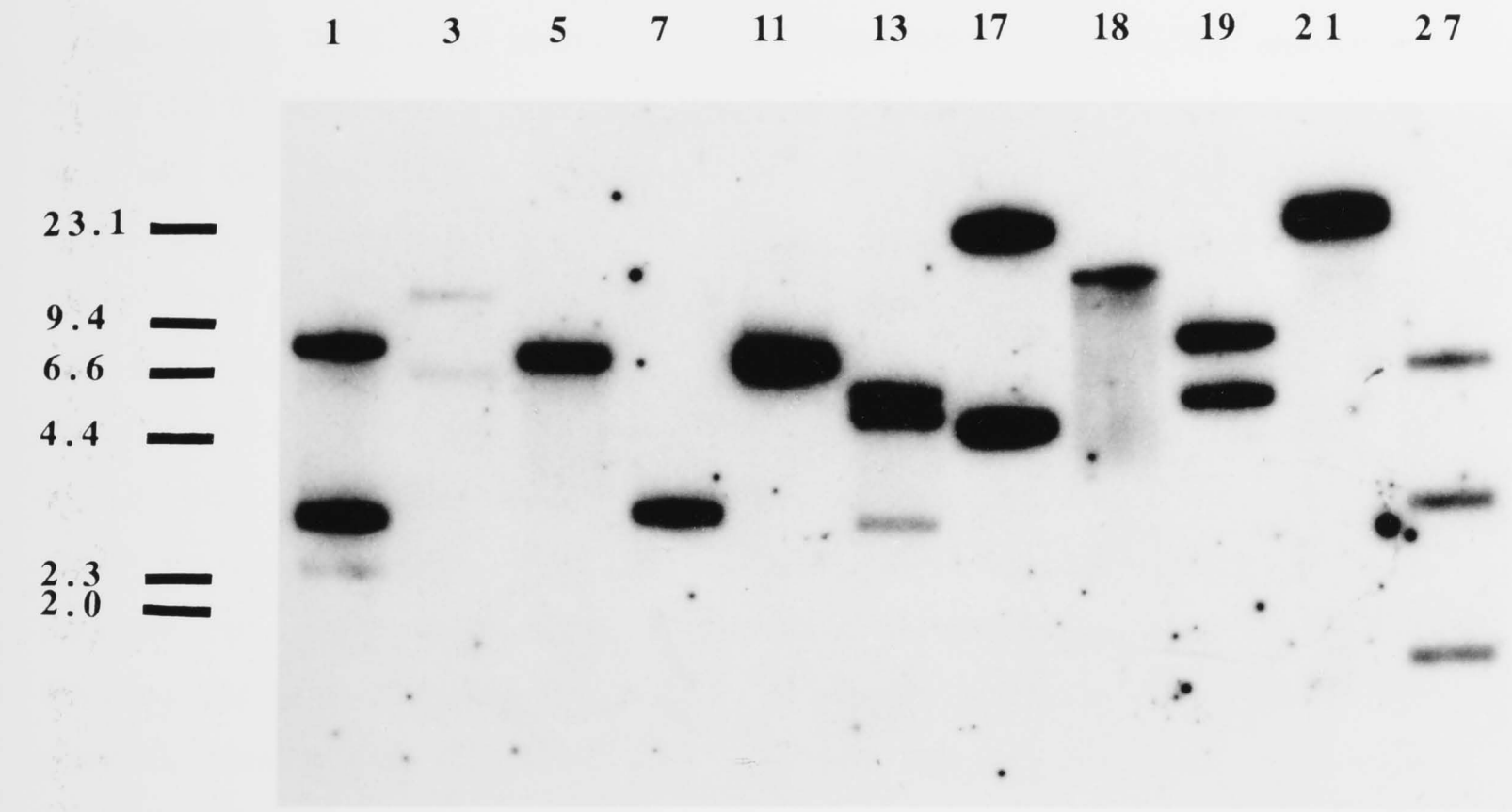


Fig. 3 Southern analysis of a few selected *Hind*III-digested λ clones using the LTR probe. Lane numbers refer to the λ-clone designation. DNA size markers are indicated.

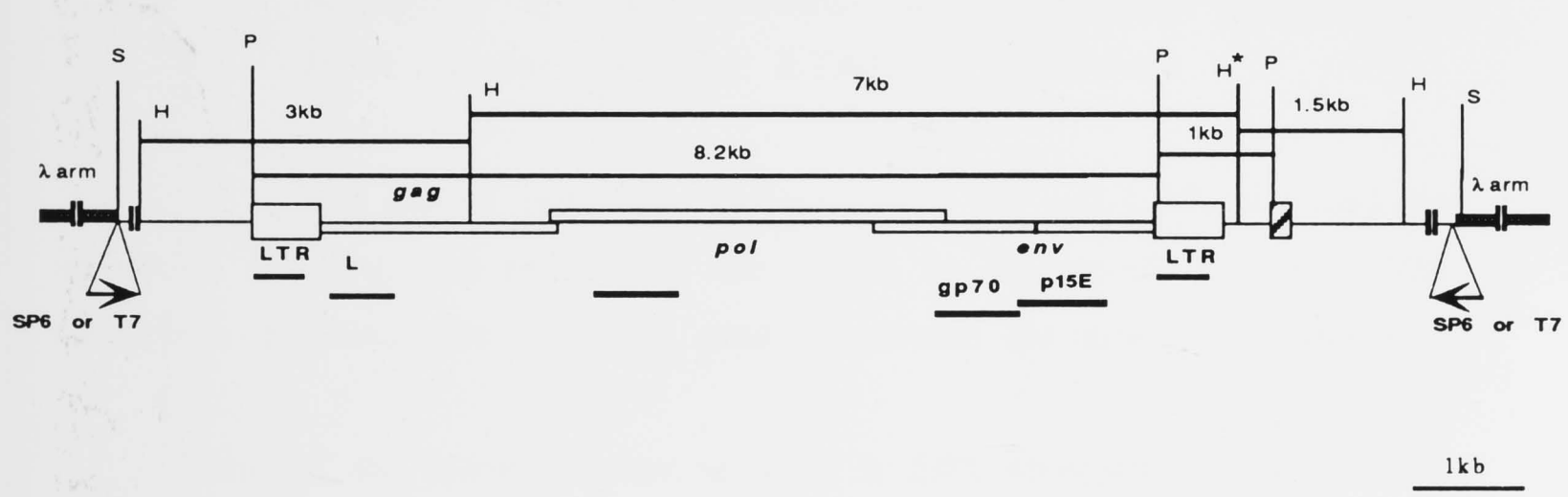


Fig. 4 Hypothetical partial restriction map of λ-27. This was based on the sequence of the 1.5kb *Hind*III LTR fragment, as well as the assumption that *Pst*I cuts at each LTR to generate a normal 8.2kb full length virus fragment. The *Hind*III site (H*) was confirmed by sequencing to be 250bp from the *Pst*I site in the LTR structure (▨). Hybridisation with the LTR probe would give 3 fragments, namely 7.0kb, 3kb and 1.5kb, as depicted (See also Fig. 3). SP6 and T7 RNA polymerase transcription initiation sites at the arms are indicated. RadLV probes used to identify viral fragments are indicated by bold black lines. The restriction enzymes used were S(*Sfi*I), H(*Hind*III) and P(*Pst*I). Mouse genomic flanking sequences are represented by a single line. The orientation of provirus relative to λ arms could not be determined as *Hind*III sites occur at both vector/insert junctions.

validity of a third LTR structure. Comparison with murine LTR sequences using the BLAST utility indicated that this LTR structure is only 180bp in size, and the *Hind*III site is approximately 250bp from the *Pst*I site in the LTR. Southern analysis of pλ-27f(C)1 and sequencing indicated that no other viral genes are present (data not shown). Based on these results and the sizes of viral fragments generated by digestion of λ-27 with *Hind*III and *Pst*I, it was concluded that *Hind*III could not cut internally at one or both LTRs. The lack of a third LTR fragment with *Pst*I digestion could be due to another *Pst*I site in close proximity, generating a small fragment of undetectable size. A hypothetical restriction map of λ-27, showing the positioning of the 3 LTRs is shown in Figure 4. However, the possible presence of this third LTR structure can be confirmed only with further subcloning and sequencing of the fragments carrying the LTR-host junction. λ-13 has not been mapped due to difficulties with subcloning. The possibility that λ-13 and λ-27 are both cloning artefacts, and that a third LTR structure is not present in the *in situ* situation has not, as yet, been excluded.

5.3.2 Mapping of viral clones and flanking genomic DNA distal to the LTR/host junction

The partial restriction maps of λ-1, λ-7 and λ-17, deduced by restriction mapping and sequencing are shown in Figures 5, 6 and 7. The restriction endonuclease *Sfi*I was used to estimate the size of the inserts.

None of the inserts appear to carry a *Sfi*I recognition site except λ-1 (Fig. 5). The size of the insert in λ-1 was estimated to be approximately 20.2kb. Restriction sites at the 3' end of the insert in λ-1 were determined by sequencing of the LTR fragment, pλ-1f1(H), and direct sequencing of λ-1 from the T7 primer site at the left arm (Fig. 5). The *Kpn*I sites were deduced from restriction mapping of pλ-1f1(H). The *Hind*III site in the genomic DNA adjacent to the 5'LTR is approximately 100bp from the *Pst*I site in the LTR. This was confirmed by the subcloning and sequencing of pλ-1f2(H).

The physical map of λ-7 and positioning of the viral/genomic DNA insert was deduced from restriction mapping with *Hind*III, subcloning of

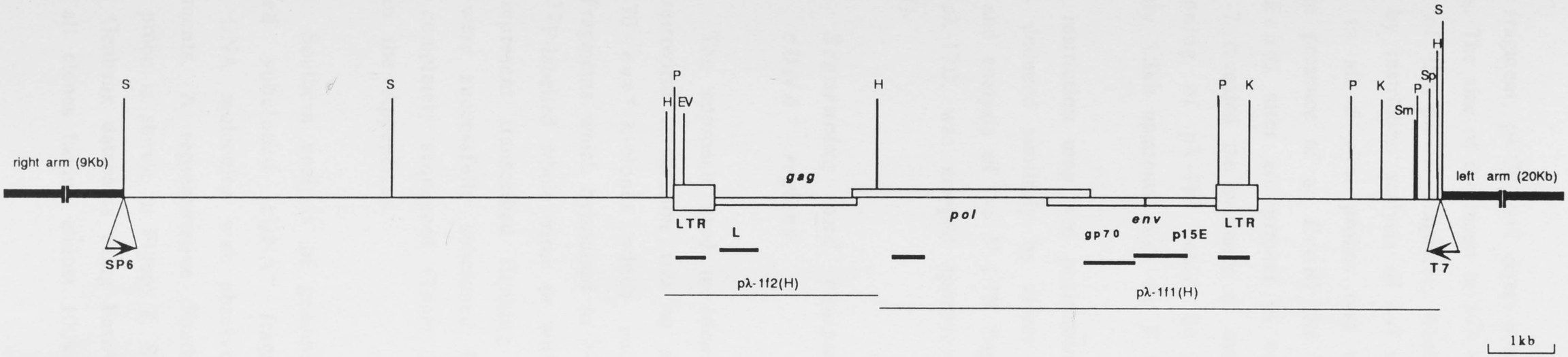


Fig. 5 Partial restriction map of λ-1 showing the position of the full length virus and flanking genomic DNA. The SP6 and T7 RNA polymerase transcription initiation sites at the right and left arm, respectively are indicated. The RadLV probes used to identify viral fragments are indicated by bold black lines. The restriction enzymes used were S(*Sfi*I), H(*Hind*III), E(*Eco*RI), P(*Pst*I) and *Kpn*I (K). *Sph*I (Sp), *Eco*RV (EV) and *Sma*I (Sm) sites were mapped by sequencing either λ-1 using the T7 primer site at the left arm or the 3' and 5' LTR fragments [pλ-1f2(H) and pλ-1f1(H), respectively], which had been subcloned into pGEM^R vectors. Genomic regions are represented by a single line. The sizes of the right and left λ-arms are indicated.

the 5'LTR fragment, pλ-7G and direct sequencing of λ-7 using the T7 and SP6 primers. The size of the insert in λ-7 was estimated to be ~16.4kb. The positioning of the pλ-7K fragment, which is a cDNA$^+$ fragment, was determined by restriction analysis of λ-7 with *Eco*RI. A fragment ≥18.0kb hybridising to all 5 viral probes was obtained. Sequencing of pλ-7K revealed the presence of an *Eco*RI site 747bp from the 5' *Hind*III site. Since no *Eco*RI sites are present in vector or provirus, and *Eco*RI-digested λ-7 revealed the presence of only one *Eco*RI site in the insert, the positioning of pλ-7K could be accurately determined to be approximately 5.3kb upstream from the 5' LTR (Fig. 6).

The restriction map and positioning of λ-17 with respect to the vector was deduced similarly by direct sequencing of λ-17 as well as subcloning and analysis of the 5' LTR fragment, pλ-17f2(D). The cDNA$^+$ fragment, pλ-17D, was mapped approximately 1.8kb upstream of the 5' LTR (Fig. 7).

5.3.3 Sequencing and computational analysis of cDNA$^+$ regions

The approach used to identify new genes expressed as a result of retroviral integration was to identify restriction fragments of RadLV gp70 *env*$^+$ λ-clones which encode sequences transcribed in C1-V13D. Fragments which hybridised to ^{32}P-labelled cDNA from C1-V13D but not to ^{32}P-labelled whole virus or wild type λ (data not shown) were taken to represent transcribed flanking genomic DNA sequences. Six fragments were successfully subcloned from 6 different λ-clones and four were completely sequenced (Table 3). Some of the sequences are presented in the Appendices.

Southern analysis of genomic DNA was carried out using ^{32}P-labelled subcloned cDNA$^+$ fragments as probes. A smear of unresolved DNA molecules was observed, indicating the presence of repeat elements. A representative Southern blot using ^{32}P-labelled pλ-17D as probe is shown in Figure 8. Sequencing and comparison to the EMBL and Genbank databases using FastA and BLAST programs revealed regions in all clones having almost 100% homology with mouse B1 and

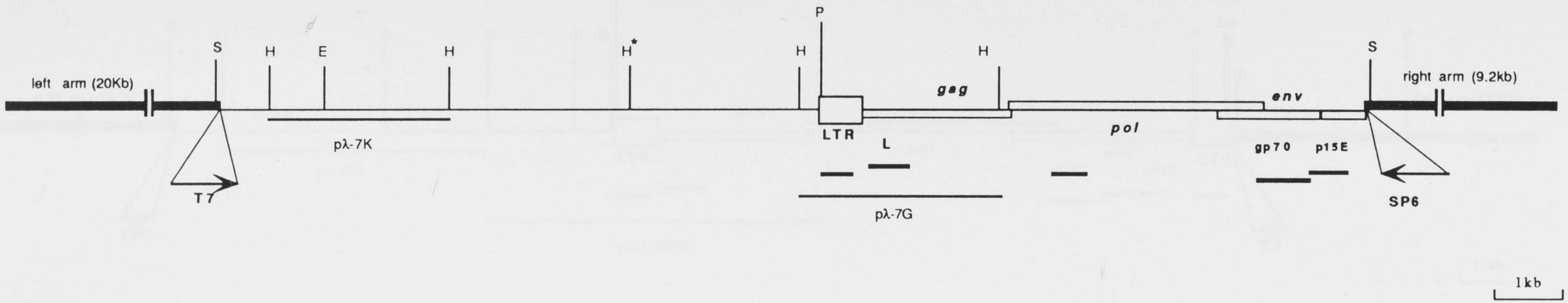


Fig. 6 Partial restriction endonuclease map of λ-7, showing the positions of the provirus and flanking genomic sequences. The SP6 and T7 RNA polymerase transcription initiation sites are indicated. RadLV probes used to identify viral sequences are indicated by bold black lines. The position of the subcloned transcribed region, pλ-7K and the subcloned 5'LTR fragment pλ-7G are shown. The restriction enzymes used were S(*Sfi*I), H(*Hind*III), E(*Eco*RI) and P(*Pst*I). The position of H* is not definite and could either generate a fragment of 2.2kb or 2.6kb with its neighbouring left or right *Hind*III site. Genomic DNA is represented by a single line. The sizes of the right and left λ-arms are indicated.

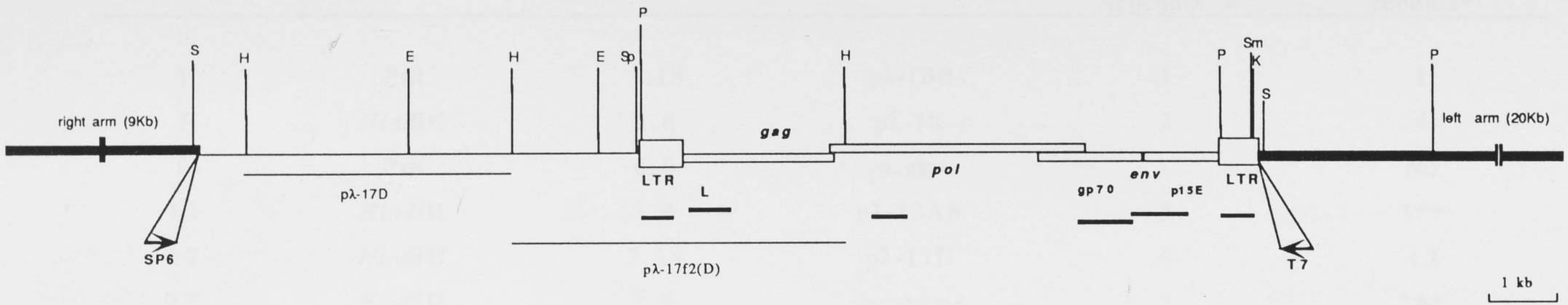


Fig. 7 Partial restriction map of λ-17. The SP6 and T7 RNA polymerase transcription initiation sites are shown. RadLV probes used to identify viral fragments are indicated by bold black lines. The position of the subcloned transcribed region, pλ-17D is shown. The restriction enzymes used were S(*Sfi*I), H(*Hind*III), E(*Eco*RI), P(*Pst*I) and Sp(*Sph*I). The *Kpn*I(K) and *Sma*I(Sm) sites were mapped by sequencing the λ-17 clone using the T7 primer site at the right arm. They are consistent with those present in the RadLV LTR. The 5' LTR was cloned as a 4.8kb *Hind*III fragment into the pGEM7Zf(+) vector and designated as pλ-17f2(D). Mouse genomic flanking sequences are represented by a single line. The sizes of the right and left λ-arms are indicated.

TABLE 3 Fragments comprising cDNA+ regions successfully subcloned into pGEM^R vectors.

λ-clones	Enzyme used	Fragment size (kb)	Designation of clone	Sequence shown in Appendix #	Number of B1/B2 repeats*
1	*Pst*I	1.18	pλ-1BB	1	1
7	*Hind*III	2.6	pλ-7K	2	4
8	*Pst*I	6.0	pλ-8K+	-	ND
13	*Hind*III	2.4	pλ-13AB	3	1**
17	*Hind*III	3.84	pλ-17D	4	12
27	*Hind*III	2.6	pλ-27BD+	5	1**

* Values represent the number of regions within the fragment which are homologous with either B1 or B2 repeats.

\+ Only partial sequence has been obtained.

** These fragments have also di-nucleotide and imperfect tandem repeats.

ND Not determined.

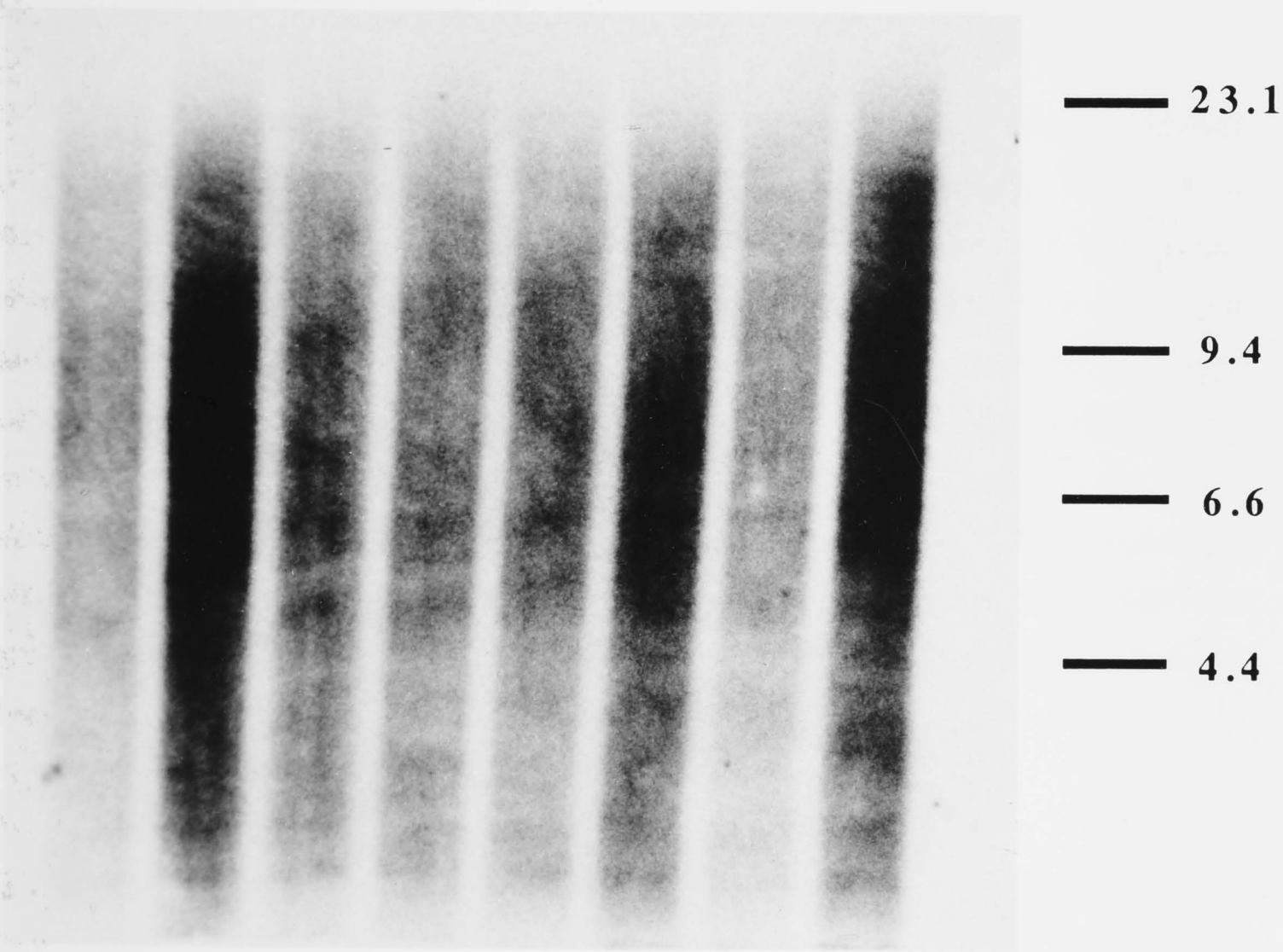


Fig. 8 Southern analysis using ^{32}P-labelled pλ-17D as a probe on *Eco*RI-digested DNA from C1-V13D and progeny clones derived following intrathymic passage of C1-V13D into CBA/H mice. CBA/H liver (LV) DNA was included as control. DNA size markers are indicated.

B2 repetitive sequences (data not shown). Some of these repeats possess poly (A) tracts. The B1 and B2 repeats, as well as stretches of dinucleotide (CA) and tandem repeats (pλ-27BD, pλ-13AB) have been highlighted in the sequences listed in the Appendices. In particular, pλ-7K and pλ-17D contained multiple regions with homology to B1 and B2 repeats (4 and 12 repeats, respectively; Table 3). Simple repeats like $(CAG)_n$, $(GTG)_n$ and (CTG) in mature transcripts of genes have been shown to be unstable and associated with disease (reviewed by Epplen *et al.*, 1993). The significance of the imperfect tandem repeats $(GAAG)_n(GA)_n$ and the $(CT)_n(CA)$ repeats present in pλ-27BD is at present unknown.

Both pλ-7K and pλ-17D cDNA$^+$ regions have been positioned 5' to the virus in the partial maps of λ-7 and λ-17, respectively (Figs. 6 & 7). The cDNA$^+$ fragment, subcloned as pλ-1BB could not be positioned exactly in λ-1, but was mapped also upstream of the virus. pλ-1BB has been subcloned as a *Pst*I fragment. Its position was deduced on the basis of elimination. It does not contain any *Kpn*I or *Sma*I restriction sites, which distinguishes it from a *Pst*I fragment of approximately the same size downstream of the virus. Since it also contains no LTR sequences, pλ-1BB cannot be downstream of the virus (Fig. 5). Clones pλ-13AB and pλ-27BD could not be positioned, since partial restriction maps of λ-13 and λ-27 could not be constructed.

5.4 Discussion

Restriction analysis has been used to confirm the presence of multiple recombinant and defective ecotropic proviruses in the C1-V13D genome. Several full length viral genomes have been cloned, including λ-7, and were shown to possess an internal *Hind*III site either 5' to the *pol* probe and 3' to the L probe, or 3' to the *pol* probe (Tables 1 & 2). No unaltered RadLV/V13 proviruses appeared to be represented amongst the λ-isolates carrying full length proviruses. All, however, hybridised to the specific RadLV gp70 *env* probe. Many of the proviral clones isolated were found to be truncated, probably as a result of the *Sau*3A1 digestion used to make the library. It is possible that one of these could represent the input RadLV/V13 virus, cloned by Rassart *et al.* (1986).

To date, there has been no report of a cloned RadLV virus containing a *Hind*III site. The virus present in λ-7 (Fig. 6) could represent recombination between the RadLV *env* sequence and an endogenous ecotropic virus in DBA/2j mouse which has a *Hind*III site in the *gag/pol* sequence. This is consistent with earlier data which demonstrated the presence of at least one endogenous virus in DBA/2j mouse which carries an internal *Hind*III site (Chapter 4, Figs. 3c, 4c & 5c). The other full length viruses cloned in λ-1 and λ-17 could also represent recombinants between endogenous viral sequences in DBA/2j mice having an internal *Hind*III site and RadLV *env* sequences.

In view of the fact that numerous truncated proviral clones were isolated, a genomic library constructed with *Eco*RI or a less frequent cutter, which does not cut the provirus internally, may have been a better option. However, *Sau*3AI was used with the objective of studying genes in the vicinity of retroviral integration. Since *Sau*3AI digestion can generate overlapping clones, the library constructed using this enzyme will be useful for future isolation and characterisation of complete genes.

The subcloning of LTR-host junctions proved to be difficult for some of the viral clones. The LTR-host junction fragments pλ-1f1(H), pλ-17f2(D) and pλ-27f(C)1 required further subcloning for complete sequence analysis. The exception was pλ-7G, which could be easily deleted by exonuclease III to generate overlapping fragments for sequencing. Restriction and partial sequence analyses of successfully subcloned LTR-host junctions have been used to deduce the composite maps of three λ-clones, namely λ-1, λ-7 and λ-17.

The subcloning of cDNA$^+$ regions using ^{32}P-labelled cDNA from C1-V13D was also undertaken. ^{32}P-labelled cDNA was used to determine if there are any coding genomic DNA sequences in the vicinity of retroviral integration. A feature of these cDNA$^+$ fragments is the presence of B1, B2 and other tandemly repeated sequences. These are interspersed abundantly in λ-7K, λ-17D and λ-27BD. The rodent B1 and B2 repeats are members of SINES (short interspersed repeat sequences) and are equivalent to the *Alu* repeats in humans (reviewed by Weiner *et al.*,

1986). The B1 repeat elements are approximately 70 - 300bp long, while the B2 repeats are typically about 190bp long (Grigoryan *et al.*, 1985). A 3' terminal oligo(A) tract sequence occurs in almost all SINES, and varies from 4 to >50bp. Although the A-rich sequence is usually a pure homopolymer tract, it is sometimes supplemented with or replaced by simple sequences such as (NAx)y (Weiner *et al.*, 1986). Some of the repeat sequences identified in the cDNA$^+$ fragments, ranging in length from 68bp to 384bp, also contained these poly(A) tracts (Appendix 1 - 5).

Single copy DNA in eukaryotic genomes has been reported to be interspersed with many reiterated sequences. These include LINES (long interspersed repeat sequences), SINES and the simple tandem repeats or repeat blocks comprising di- or trinucleotides. LINES are about 6 - 7kb long (Weiner *et al.*, 1986). Both LINES and SINES are also designated as mobile genetic elements or non-viral retroposons because of their ability to move from one genetic locus to another via an RNA intermediate. They share this property with the retroviruses, *copia* elements in Drosophilia and Ty elements in yeast (Shapiro, 1983). There have been a number of reports on transposition of these elements into oncogenes and other structural genes. As a result, the activities of these genes were affected (Katzir *et al.*, 1985; Arman *et al.*, 1986, Morse *et al.*, 1988).

There is increasing evidence to indicate that these repetitive sequences interspersed in the eukaryotic genome are not just 'junk' DNA. Some of them have been shown to have specific regulatory roles and to code for functional proteins (Davidson & Britten, 1979; Laimins *et al.*, 1986; Mathias *et al.*, 1991). For example, SINES have been shown to carry a functional internal RNA polymerase III promoter and have been implicated as a possible origin of DNA replication and/or RNA polymerase III transcription (Weiner *et al.*, 1986). SINES have also been proposed as tissue-specific markers; examples are the rat ID (or identifier) sequence, which has been proposed as a brain-specific transcript marker (Sutcliffe *et al.*, 1984, Weiner *et al.*, 1986) and mouse SET 1 (or B2) sequences, which has been proposed as a marker for mRNAs specific to both normal embryonic and oncogenically transformed cells (Murphy *et al.*, 1983; Scott *et al.*, 1983). Transcription

levels of B2 sequences have been observed to be elevated in tumour cells and it was suggested that this may be related to tumour progression (Grigoryan *et al.*, 1985). $(CA)_n$ blocks have been associated with sites of recombinational events (reviewed by Rogers, 1983) and alterations in CA repeats have been shown to occur in human gastric and colonic cancers (Mironov *et al.*, 1994). Expansion of simple di- or trinucleotide repeats from a few to hundreds or more have also been shown to be associated with a number of inheritable genetic diseases (Kunkel, 1993). Also, certain $(GT)_n$ or mixed $(GT)_n/(GA)_n$ stretches of intronic simple repeats are shown to be preserved in immunologically relevant genes and they bind nuclear protein molecules with high affinities (Epplen *et al.*, 1993).

The role of repeats as transcriptional silencers has also been documented. Fanning *et al.* (1985) demonstrated that the location of the MMTV-8 provirus within a repeat region inhibited viral transcription. As a result, little or no MMTV-specific RNA can be detected in the mammary gland. However, the virus is transcriptionally active when cloned and transfected into mouse cells, suggesting that transcriptional inactivity of the provirus was due to an inhibitory effect exerted by the flanking genomic sequences. Another example of repeat elements inhibiting transcription was provided by Youssoufian & Lodish (1993). The authors demonstrated that transcription of the murine erythropoietin receptor (EpoR) gene is inhibited by a repetitive element located upstream of the EpoR promoter, and that the inhibitory effect is both distance and orientation dependent. The mouse EpoR gene has been shown to undergo transcriptional activation upon differentiation of embryonic stem cells *in vitro*.

The number of B1 and B2 repeats present in pλ-7K and pλ-17D (4 and 12, respectively, Table 3) appeared to be higher than the frequency estimated over the whole genome. This has been estimated to be 1 per 3×10^4 bp in the mouse haploid genome (Weiner *et al.*, 1986; Grigoryan *et al.*, 1985). The frequency of repeats in pλ-7K and pλ-17D was much higher, namely 1 per 650bp and 1 per 320bp, respectively. Furthermore, all full length viruses subcloned were found to be integrated in regions possessing repeat elements. Taken together, the

data suggested that repeat regions may represent 'hot spots' or preferred sites for retroviral integration.

LINES and SINES have been shown to be transcribed (Economou-Pachnis *et al.*, 1985; Young *et al.*, 1982; Skowronski & Singer, 1985; Weiner *et al.*, 1986). Since there are probably many of these reiterated sequences present in cDNA, it is not possible to determine if the repeats identified in the subcloned cDNA$^+$ fragments are actually transcribed in C1-V13D. These fragments will therefore be simply referred to as cDNA$^+$ fragments or regions in this study.

The presence of repeat sequences in some of the cDNA$^+$ fragments has made the search for putative open reading frames and coding regions difficult. Computational analysis has revealed no other significant homology with sequences in the nucleic acid databases, indicating that the repeats could either be present in purely intronic regions, or that they could represent novel sequences (data not shown). Some small stretches of putative coding sequences present between repeat elements, and in some cases encompassing some of the repeat sequences, were indicated by computational analysis (data not shown). These findings will need to be confirmed by isolating the corresponding cDNAs.

In view of the evidence that repeat elements may have regulatory roles and are highly mobile genetic elements, it is possible that retroviral integration into the vicinity of repeat elements could result in tumorigenesis either by a) disruption of any normal regulatory role attributed to sequences in the repeat, or b) increased instability of these elements, leading to enhanced retropositioning.

SUMMARY

A genomic library of C1-V13D has been constructed and λ clones were selected for their capacity to hybridise with the specific RadLV gp70 *env* probe. Analysis of these clones by a combination of approaches, including subcloning, partial restriction mapping and sequencing, has

confirmed the existence of multiple recombinant and defective viruses in C1-V13D. To check for the presence of coding genomic sequences, ^{32}P-labelled cDNA from C1-V13D was used to probe *Hind*III- and *Pst*I-digested virus-positive λ clones by Southern analysis. Transcribed or cDNA$^+$ regions, not hybridising to ^{32}P-labelled wild type λ and virus sequences, were subcloned and analysed. A notable feature of these cDNA$^+$ regions was the frequent presence of B1, B2 and simple repeats, which made attempts to identify other possible coding regions very difficult. Some of these sequences were shown to be present in significantly higher frequency in λ clones than in the genome as a whole. All of the λ clones carrying full length viruses appeared to be integrated in regions possessing repeat elements. Since some B1 and B2 repeats have been documented to code for functional proteins and to play regulatory roles, it is tempting to hypothesise that viral integration next to these mobile genetic elements could provide a mechanism for oncogenesis via disruption of their normal gene regulatory function. In addition, these elements are genetically unstable and integration of the virus could cause further genetic instability, resulting in enhanced transposition.

CHAPTER 6

Sequence analysis of the 5'LTR-host junction in λ-7

6.1 Introduction

The analysis of LTR-host junctions and common integration sites has led to the discovery of cellular genes disrupted by retroviral integration (Bergeron *et al.*, 1991; Sels *et al.*, 1992; Tremblay *et al.*, 1992). In rodents, the *c-myc*, *Pim-1*, *Mlvi-1*, *Mlvi-4*, *Tp-1* genes have been identified as common MuLV provirus integration sites (reviewed in Section 1.5.3). Their rearrangements have been found to correlate with development and progression of T cell leukemia. In Chapter 5, analysis of $cDNA^+$ regions in the vicinity of retrovirus integration has revealed the frequent presence of repeat elements.

In this study, a few LTR/host junction DNA were subcloned successfully. One of these, namely pλ-7G was completely sequenced. pλ-7G contained the 5'LTR and a small stretch of adjacent host DNA sequences (~253bp) subcloned from λ-7 (Chapter 5, Fig. 6). This chapter was focused on the characterisation of pλ-7G. The objectives were to (1) identify any potential new gene and (2) determine if this gene, which corresponded to the flanking genomic sequence, was rearranged and expressed differentially in C1-V13D compared to germline DBA/2j and other cell types. Another objective was to determine if this site of retroviral integration was common to other lymphoid tumour lines induced by RadLV. Finally, attempts were made to characterise this flanking genomic sequence using the ANGIS network to determine if there was any significant homology with both nucleotide and amino acid sequences recorded in the GENBANK, EMBL, SWISS-PROT and GENPEPTIDE databases. It was anticipated that since C1-V13D represents an immature, partially transformed lymphoid cell line, the isolation of any new genes

in the vicinity of retroviral integration may give an insight into the transformation process or early differentiation of lymphoid cells.

6.2 Materials and Methods

6.2.1 Derivation of λ-7

The derivation of λ-7 and DNA preparation are described in Sections 5.2.1 - 5.2.3. The composite map is shown in Figure 6, Chapter 5. The 3'LTR has been truncated by *Sau*3AI during library construction. Restriction mapping and Southern analysis using all viral probes have indicated that it is unlikely to be a defective virus.

6.2.2 Subcloning of the 5'LTR-host junction from λ-7

The 2.55kb *Hind*III fragment, comprising the 5' LTR-host junction, was subcloned into the pGEM7Zf(+) plasmid at the *Hind*III polycloning site. The procedure for subcloning has been described in Section 4.2.4. The orientation of this fragment within the plasmid is depicted in Fig. 1b. It has been designated as pλ-7G (Chapter 5, Fig. 6).

6.2.3 Analysis of flanking genomic DNA immediate to the LTR

6.2.3A Unidirectional deletion of pλ-7G using ExoIII

The procedure for unidirectional deletions, which is used to generate overlapping smaller fragments for sequencing, has been described in Section 5.2.5C. The pλ-1f1(H) and pλ-17f2(D) clones, carrying LTR/host junction fragments, were not *Exo*III deleted either because a) there were no enzymes that could generate protective 3' overhangs without cutting the insert as well, or b) there were no suitable enzymes to generate 5' overhangs. The pλ-7G clone was easily *Exo*III deleted and therefore sequenced using this method. *Sph*I was used to generate the exonuclease III resistant 3' overhang and *Eco*RI to generate the 5' overhang. Twelve time points, four at 1 minute intervals

and eight at 30 seconds intervals were taken. The deletion-containing plasmids were then ligated to re-circularise and transfected into *E. coli* hosts using the heat shock method as described in Section 4.2.4B.

6.2.3B Cycle sequencing of pλ-7G ExoIII deletions with dye primers

Cycle sequencing using dye primers has been described in Section 5.2.4B. The T7 dye primer was used since the deletions proceeded from the T7 transcription start site.

6.2.3C Southern analysis

The procedure for Southern analysis is described in Section 2.2.8. The 7G probe was used. It is a 297bp fragment amplified from pλ-7G using the 7GR (5' AGG GAG TGG GCA GGA GAA) and the SP6 primers (5' ATT TAG GTG ACA CTA TAG). Preparation of this probe was necessary because there were no suitable enzymes to completely eliminate the adjacent LTR sequences. The 7GR primer was designed from the genomic DNA sequence in the λ-7G clone (Fig. 1a) using the Oligo (Wejciech Rychlik, © 1991) and Amplify version 1.0 (Bill Engels, © 1992, Wisconsin University, Genetics, USA) programs. Amplification was carried out in a standard 3-step cycling reaction comprising an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 15 seconds, 48°C for 15 seconds and 72°C for 45 seconds in the FTS-1 thermal cycler (Corbett Research, Australia). Amplification was performed twice. The first amplification was carried out in a 20µl reaction containing 20ng DNA template, 7.5pM of primers OSP6 and 7GR, 0.2mM dNTP (Pharmacia, USA), 0.2U Super Taq enzyme in 1X Super Taq buffer (P.H. Stehelin & CIE AG, Basel, Switzerland). The amplified probe was electrophoresed through 1% GTG SEAKEM agarose (FMC BioProducts, USA) and electroeluted. The second amplification was performed under the same conditions using the first amplified product (10pg) as the template. This final preparation was electrophoresed, excised and extracted from the gel using the Geneclean kit (BIO 101, USA).

a)

```
   1 CAATCATAAT GAAGTGGCAC GACAGGTTCC CGACTGGAAA GCGGGCAAGT GACCGAACGC AAATTAATGT GAGTAGCTCA CTCATTAGGC ACCCCAGGCT

 101 TTACACTTTA TGCTTCCGGC TCGTATGTTG TGTGGAATTG TGAGCGGATA ACAATTTCAC ACAGGAAACA GCTATGACCA TGATTACGCC AAGCTATTTA
          SP6 →
 201 GGTGACACTA TAGAATACTC AAGCTATGCA TCCAACGCGT TGGGAGCTCT CCGGATCCAA GCTTCTTCCT CCTCCTCCTC CTCTTCCTCC TCCTCCTCCT

 301 CTTGTCTCCT CTTCTGTCTC CTCTTCCTTC TCCTCCTCTT CCTCCTCTTG CTCCTCCTTT CCCTCCTCCT TCTTTATTCC AAACAGGTTG GGNCTATGCT
                                                                    ← 7GR
 401 GTTCAGGCTG GCTTAGGACT CACTATGTAG CCCACGCTAG ACTTGAATCT GCTATGAACC TTCTGCCTCA GCTTCTCCTG CCCACTCCCT ACTGAAAGAC

 501 CCCTTCATAA GGCTTAGCCA GCTACCTGCA GTAACGCCAT TTTGCAAGGC ATGGGAAAAT ACCAGAGCTG ATGTCCTCAG AAAAACAAGA ACAAGGAAGT

 601 ACAGAGAGGC TGGAAAGTAC CGGGACTAGG GCCAAACAGG ATATCTGTGG TCAAGCACTA GGGCCCCGGC CCAGGGCCAA GAACAGATGG TCCCCAGAAA

 701 TAGCTAAAAC AACAACAGTT TCAAGAGACC CAGAAACTGT CTCAAGGTTC CCCAGATGAC CGGGGATCAA CCCCAAGCCT CATTTAAACT AACCAATCAG

 801 CTCGCTTCTC GCTTCTGTAC CCGCGCTTAT TGCTGCCCAG CTCTATAAAA AGGGTAAGAA CCCCACACTC GGCGCGCCAG TCCTCCGATA GACTGAGTCG

 901 CCCGGGTACC CGTGTATCCA ATAAAGCCTT TTGCTGTTGC ATCCGAATCG TGGTCTCGCT GAACCTTGGG AGGGTCTCCT TCAGAGTGAT TGACTGCCCC

1001 AACCTGGGGT CTTTCATT
```

b)

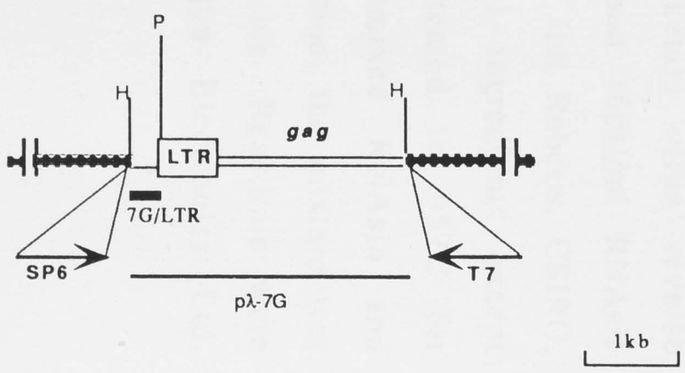


Fig. 1 Partial nucleotide sequence of a) pλ-7G and b) a very simplified map depicting its orientation in the pGEM7Zf(+) vector.

a) Only the nucleotide sequence up to the 3' end of the 5'LTR is shown here. The full nucleotide sequence is presented in Appendix 6. The boxed region represents the LTR sequence. The SP6 and 7GR primers are heavily underlined and the arrows indicate the direction of transcription. The genomic sequence immediate to the LTR is boxed and shaded. Numbers represent base pairs. The unboxed, unshaded sequence belongs to the pGEM7Zf(+) vector. The 11bp inverted repeats at the 5' and 3' end of the LTR are shaded.

b) The heavy black line represents a probe which was prepared by digesting the plasmid and insert with *Hind*III and *Pst*I. It contained approximately 30bp LTR sequence and was designated as 7G/LTR.

6.2.3D Northern analysis

The procedure for Northern analysis has been described in Section 3.2.5. RNA was prepared from C1-V13D as well as from the RadLV-induced thymic lymphoma cell lines, BL/VL3 and C6VL/1. RNA from various tissues was also prepared from CBA/H, DBA/2j and C57BL/Ka mice for comparative analysis. The probes used were 7G (PCR product, Section 5.2.6D) and 7G/LTR, an ~300bp *Hind*III/*Pst*I fragment (Fig. 1b). This probe also contains approximately 30bp of the 5'LTR sequence.

6.2.4 Strategy for isolation and analysis of C1-V13D cDNA corresponding to the genomic flanking DNA sequences in pλ-7G

6.2.4A Preparation of poly(A)$^+$ RNA and cDNA synthesis

The preparation of poly(A)$^+$ RNA from C1-V13D and DBA/2j liver (LV) was carried out as described in Section 5.2.5A. Synthesis of first strand cDNA was carried out according to Sambrook *et al.* (1989), with minor modifications. The reaction was carried out at 42°C for 60 minutes in 20µl Reverse Transcriptase buffer (Gibco-BRL, USA) containing mRNA (1 to 1.5µg) or total RNA (10µg), 3pM NOT-TT primer (5' GGG CCC ATG CGG CCG CAA TTA ATT TTT TTT TTT TTT TTT), 1mM dNTP (Pharmacia, USA), 1000U/ml Moloney murine leukemia virus reverse transcriptase (MoMuLV RT) from Gibco-BRL, USA and 100U/ml RNAsin (Promega, USA). The NOT-TT primer was a gift from Jim Roberts, CSIRO, Divison of Plant Industry, Canberra, Australia. All ingredients except RNAsin and MoMuLV RT were premixed and heated to 95°C for approximately 2 minutes and cooled to 42°C. Premixed RNAsin and MoMuLV RT was then added. At the end of the reaction, the mixture was heated to 95°C for 5 minutes before chilling on ice. Reactions were carried out on the Cambio thermal cycler (Cherlyn Electronics Ltd, Cambridge, England).

6.2.4B Amplification of "7G-cDNA" and DBA/2J liver cDNA using PCR

cDNA prepared as described above was amplified using NOT-TT and 7GR as reverse and forward primers, respectively. The amount of cDNA template used was equivalent to that synthesised from 50ng poly(A)$^+$ RNA or from 1mg total RNA. Two rounds of amplification were carried out. The first amplification reaction mix comprised 5pM 7GR and 3.6pM NOT (5' GCG GCC GCA ATT AAT TT) primers, 0.2mM dNTP and 0.2U Super Taq enzyme in 1X Super Taq buffer. The total reaction volume was 18µl. The reagents (primers, dNTP and template) were premixed and heated to 94°C before the addition of enzyme and buffer. The cycling parameters were 5 cycles of 94°C for 15 seconds, 54°C for 30 seconds and 72°C for 2 minutes, followed by 40 cycles of 94°C for 15 seconds, 50°C for 30 seconds and 72°C for 2 minutes. The amplified material was diluted 100X. This was used as the template for re-amplification using NOT-TT as the reverse primer. The protocol comprised 40 cycles of 94°C for 15 seconds, 54°C for 30 seconds and 72°C for 2 minutes. The reactions were carried out in the FTS-1 thermal cycler (Corbett Research, Australia). The products were electrophoresed on 1.5% low melting temperature Nusieve GTG agarose gel (FMC BioProducts, USA), excised, extracted using the Magic PCR preparation kit (Promega, USA) and stored at -20°C.

6.2.4C Cycle sequencing of "7G-cDNA" with dye terminators

Cycle sequencing of PCR products was carried out using dye terminators with NOT-TT and 7GR as primers. Sequencing (25 cycles) was performed in a 20µl reaction mix containing 9.25µl Dyedeoxy Terminator premix (Applied Biosystems Inc, USA), 3.2pM primer and 0.2ng/bp DNA template in sealed capillary tubes in the FTS-1 thermal cycler (Corbett Research, Australia). Cycling parameters were a) denaturation at 96°C for 30 seconds, b) 54°C for 15 seconds and c) 60°C for 5 minutes. At the end of the sequencing reaction, the mixture was adjusted to 100µl with dd$H_2$0, extracted twice with phenol/chloroform mix (ABI, USA) and the sequenced product was precipitated with 0.1X volume 3M sodium acetate/pH 5.2 and 300µl of ethanol for >15 minutes on ice. It was pelleted,

washed and vacuum dried. The samples were electrophoresed as described in Section 5.2.4B.

6.2.4D Computational analysis

FastA and BLAST utilities were used for sequence analysis. The BLAST utility also translated the nucleotide sequence into all six reading frames and simultaneously compared them with amino acid sequences in the protein databases (EMBL and Swiss-Prot databases).

6.3 Results

6.3.1 Rearrangement of a single gene via retroviral integration was detected in C1-V13D with the 7G probe

To confirm that the 7G probe was the correct fragment after amplification from plasmid λ-7G, it was ^{32}P-labelled and tested on the same blot as depicted in Figure 3, Chapter 5. Besides hybridising strongly to the correct λ-7 *Hind*III fragment (~3.0kb), it also hybridised strongly to the 2 smaller LTR fragments in λ-27 (Fig. 2). The smaller 1.5kb fragment, subcloned as pλ-27f(C)1 [Section 5.2.6A] was sequenced and confirmed to share 100% homology with the mouse genomic flanking sequence in pλ-7G. Since both the provirus in λ-7 and in λ-27 gave an approximately 3.0kb 5'LTR-host junction fragment, it is possible that both viruses are the same.

Southern analysis was performed to detect any genetic rearrangement or polymorphism corresponding to the pλ-7G flanking genomic DNA in various tissues and cell lines. *Hind*III-digested liver, spleen and thymus DNA from DBA/2j, CBA/H and C57BL/Ka were compared with C1-V13D DNA using the 7G probe. A single fragment was detected in all 3 strains in all the tissues examined, indicating homozygosity for both alleles. The size of this fragment was approximately 1.1kb in both DBA/2j and CBA/H while the fragment detected in C57BL/Ka was approximately 3.5kb (Fig. 3a). Polymorphism

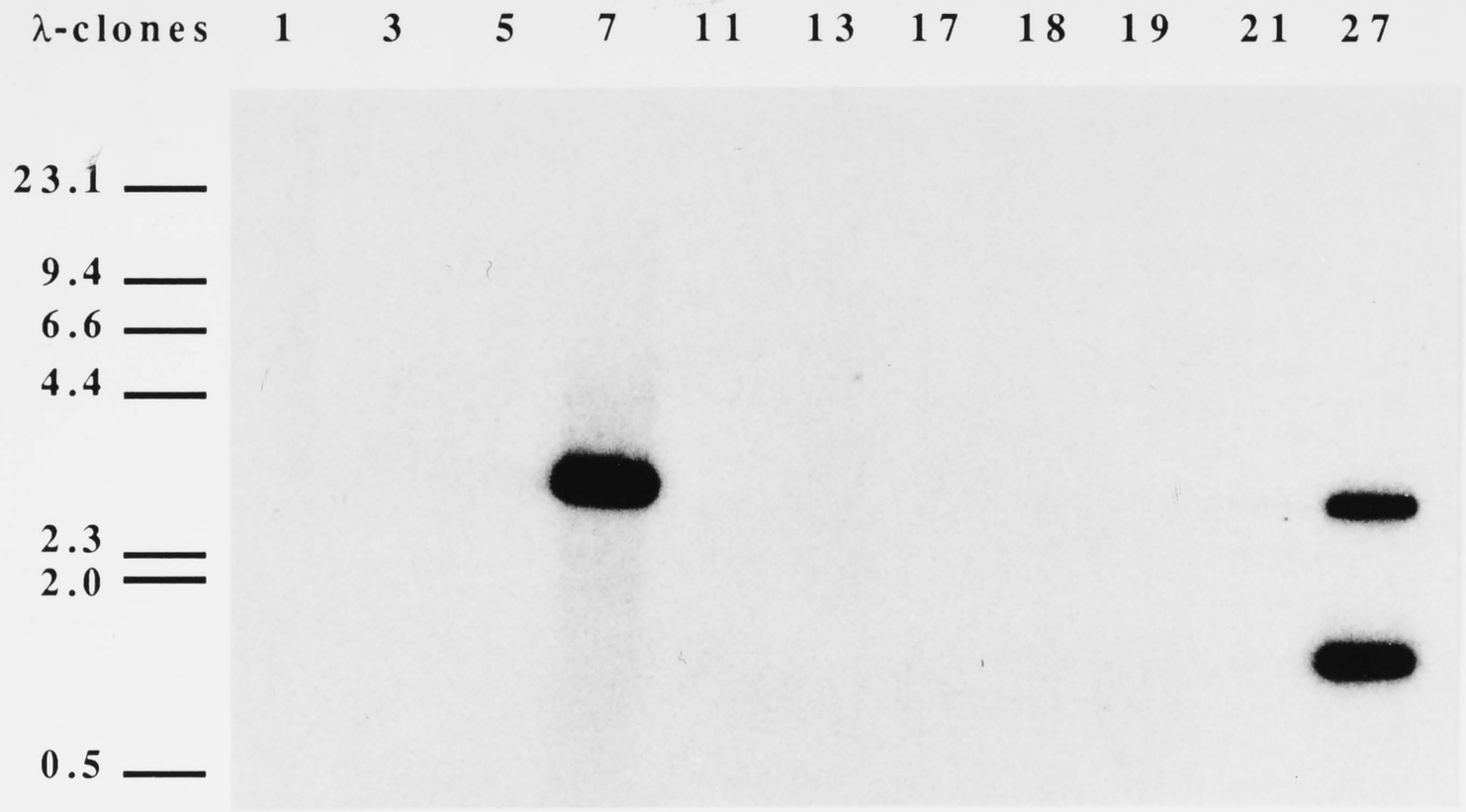


Fig. 2 Southern analysis of selected *Hind*III-digested λ-clones, using ^{32}P-labelled 7G as probe. This probe hybridises to the 2.8kb LTR fragment in λ-7 and the smaller 3.0kb and 1.5kb LTR fragments of λ-27. DNA size markers are indicated.

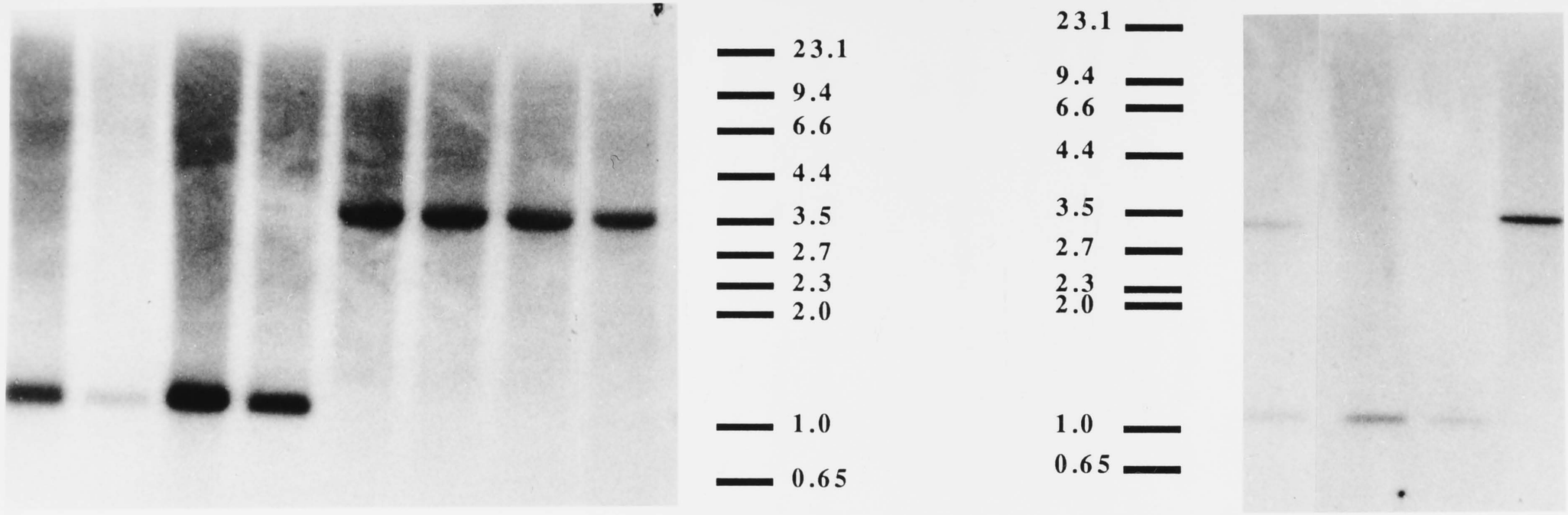


Fig. 3 Southern analysis of *Hind*III-digested DNA using ^{32}P-labelled 7G probe. a) Polymorphism was observed in the different mouse strains studied, which include syngeneic DBA/2j and allogeneic CBA/H and C57BL/Ka (Ka) mice. b) Rearrangement of the *c-7G* gene is observed in C1-V13D compared to normal adult spleen (Spl), thymus (Thy) and liver (LV) cells from different mouse strains. BL/VL3 and C6VL/1 are RadLV-induced thymic lymphoma cell lines. DNA size markers (kb) are indicated.

was therefore indicated at this locus amongst these mouse strains. C1-V13D retained the germline band of approximately 1.1kb, and there was evidence of acquisition of a new rearranged 2.8kb fragment (Fig. 3b). Both the 1.1kb and 2.8kb fragments were of equal intensity and were weaker than the germline 1.1kb fragment present in DBA/2j liver DNA control. This suggests that the virus has integrated into one allele. The rearrangement seen in C1-V13D appears to be specific for this cell line since spleen, liver and thymus DNA from syngeneic DBA/2j mouse do not possess this new band. Analysis of DNA from two RadLV-induced thymic lymphoma cell lines, BL/VL3 and C6VL/1, also showed no rearrangements compared to their germline C57BL/Ka controls. This confirmed a rearrangement unique to C1-V13D and not to all RadLV-induced tumour cell lines.

6.3.2 Tissue-specific expression of a gene homologous with the 7G probe

Northern analysis of C1-V13D revealed the presence of 3 mRNA transcripts of 1.6kb, 3.1kb and 4.6kb hybridising to the 7G/LTR probe (Fig. 4a). The 7G/LTR probe contained ~30bp of LTR sequence (Fig. 1b). The 7G probe, which has no LTR sequences, detected a 1.6kb and a 4.6kb transcript (Fig. 4b). These transcripts were detectable in C1-V13D and in its progeny clones ET4-D, ET4-D(T1-E) and ET4-D(T1-E)M7A (Fig. 4a). Analysis of this same Northern blot with the LTR probe revealed the presence of 2 fragments corresponding to the 3.1kb and 4.6kb fragments, but not the 1.6kb band (data not shown).

The gene detected here is arbitarily designated *c-7G* (*c* to represent cellular sequences). *c-7G* was observed to be also expressed by WEHI-279, a B cell lymphoma, as well as C6VL/1 and BL/VL3, which were derived from 2 different RadLV-induced T cell lymphomas. It is also expressed by adult lymphoid tissues such as the spleen and thymus from DBA/2j mice but not expressed by liver cells from adult syngeneic DBA/2j and allogeneic CBA/H mouse strains. The expression of *c-7G* therefore appears to be tissue specific, with expression confined to cells of lymphoid origin. The level of expression does not seem to be elevated in C1-V13D in comparison with the DBA/2j spleen control.

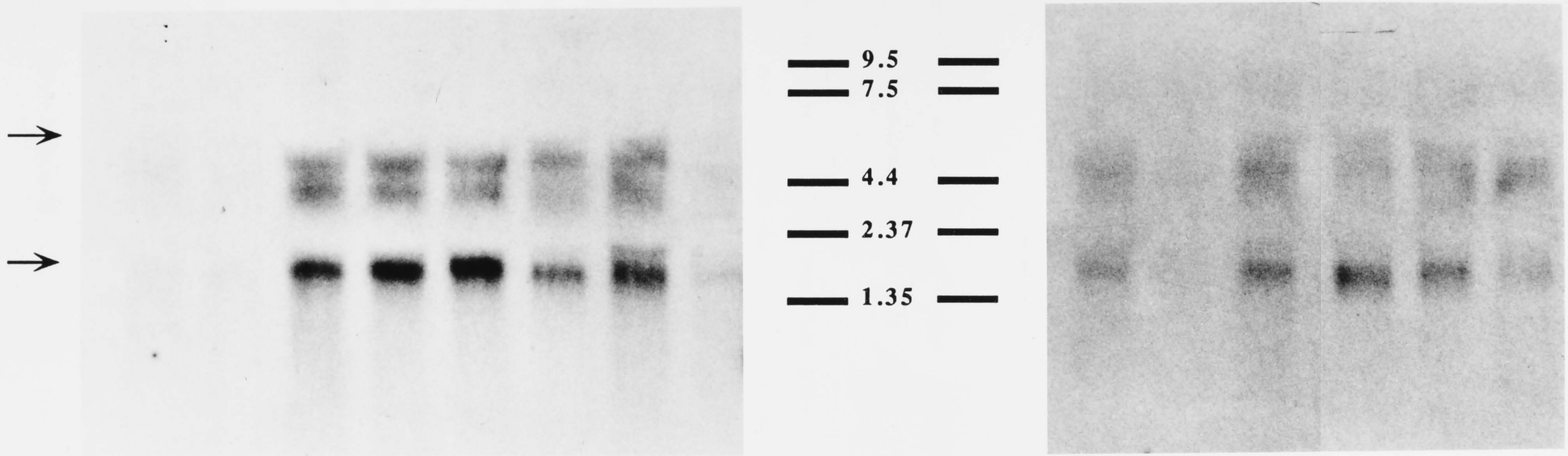


Fig. 4 Demonstration of tissue-specific expression of *c-7G* by Northern analysis. Total RNA (20mg/sample) from C1-V13D was compared to RNA from liver (LV), spleen (Spl) and thymus (Thy) tissue. RNA from T lymphoma cell lines, BL/VL3 and C6VL/1 and WEHI-279, a B cell lymphoma cell line were also included for comparison. RNA size markers are indicated. Arrows indicate the levels of migration of the 28S and 18S ribosomal RNA species. a) The RNA was hybridised to the 7G/LTR probe, which is a *Hind*III/*Pst*I fragment derived by the digestion of pλ-7G. b) The 7G probe, derived from amplification of λ-7G by PCR with the 7GR and SP6 primers was used for analysis.

6.3.3 Sequence analysis of the 5' LTR in pλ-7G

Analysis of the LTR sequence revealed the presence of two 11-base pair inverted direct repeats at the 3' and 5' end of the LTR (Fig. 1a). These inverted repeats are similar to those seen in the Moloney virus 5'LTR (van Beveren *et al.*, 1980). This feature was proposed to reflect the integration properties of a transposon. Comparison of the LTR sequence in pλ-7G with other MuLV proviral LTR sequences using the BLAST utility demonstrated approximately 98% homology with the LTR of the RadLV variant, G_6T_2 and with the endogenous BL/Ka(B) provirus, an ecotropic, non-leukemogenic virus. Further analysis also revealed that the 7G-LTR lacked the 43bp and 16bp direct repeats present in the moderately leukemogenic pMol502 and V21 BL/VL_3 viral clones (Rassart *et al.*, 1986).

6.3.4 Computational analysis of the flanking genomic DNA adjacent to the LTR

The flanking genomic DNA sequence present in λ-7G was compared with nucleic acid sequences in the GENBANK and EMBL nucleic acid databases. No significant homology was observed. When translated into all six reading frames (Appendix 7) and compared to protein sequences in the Swiss-Prot, PIR and GenPeptide databases using the BLAST utility, homology with several proteins in the database was observed in two minus reading frames. Only those which have included at least 20 amino acids for comparison and having >50% homology are shown (Table 1). The homology was found to be mainly in the region coding for a long stretch of charged glutamic acid (E) residues in the first minus reading frame (nucleotides 140 - 207), and a stretch of mixed basic and acidic amino acid residues in the second minus reading frame (nucleotides 140 - 250) [Fig. 5].

If one of these hypothetical reading frames is correct, it would suggest that a) the virus is integrated in a transcriptionally opposite direction to the 7G gene and b) this gene may code for a protein with a highly charged region. No start codon was found in either of the putative reading frames. This indicated that if the flanking region was

TABLE 1 Homology of charged amino acid residues coded by *c-7G* to protein sequences in the GENBANK database using the BLAST utility

Accession number	Protein	% homology* RF "-1" (84 a.a)	RF "-2" (84 a.a.)	Reference
X15209	Ryanodine receptor (pig)	86 (25/31)	67 (19/28)	Takeshima *et al.* (1989)
HSG9a	G9a protein (human)	70 (26/37)	-	Milner *et al.* (1993)
M61185	Glutamic acid rich protein (bovine)	67 (25/37)	55 (21/38)	Sugimoto *et al.* (1991)
HUMNUCLEO	nucleolin protein (human)	61 (21/34)	58 (14/24)	Srivastava *et al.* (1990)
ZEFZCMYC	c-myc gene product (zebra fish)	76 (19/25)	-	Schreiber-Agus *et al.* (1993)
SSCRCRNA	calcium release channel (pig)	88 (22/25)	69% (18/26)	Harbitz *et al.* (1992)
RATCSM	calmodulin-binding protein (rat)	80 (20/25)	87 (14/16)	Ono *et al.* (1989)
HSRAP74	transcription factor TFIIF (human)	72 (18/25)	59 (19/32)	Aso *et al.* (1992)
HSCENPBR	major centromere autoantigen (human)	86 (19/22)	50 (17/34)	Earnshaw *et al.* (1987) Sullivan & Glass (1991)
HAMBZBP	DNA-binding protein (rodent)	82 (19/23)	76 (16/21)	Franklin *et al.* (unpublished)
MMUBF	nucleolar transcription factor (mouse)	63 (24/38)	-	Hisatake *et al.* (1991)
S67171	U1 small nucleolar ribonucleoprotein (human)	61 (16/26)	66 (22/33)	Elisei *et al.* (1993)
MUSHSP86B	heat shock protein (mouse)	75 (18/24)	-	Hoffman & Hovemann (1988)

*: includes identical and homologous amino acids as defined by BLAST algorithm.
RF "-1": Reading frame -1 ($K_2E_2GKE_2QE_6KE_3TE_3$) RF "-2": Reading frame -2 ($R_4ER_2SKR_2KR_4KR_2QKR_2QE_{12}K$)

5' to 3' (minus strand)

```
        10        20        30        40        50        60        70
        *         *         *         *         *         *         *
CCTTATGAAGGGGTCTTTCAGTAGGGAGTGGGCAGGAGAAGCTGAGGCAGAAGGTTCATAGCAGATTCAA
  L  W  R  G  L  S  V  G  S  G  Q  E  K  L  R  Q  K  V  H  S  R  F  K>
 L  M  K  G  S  F  S  R  E  W  A  G  E  A  E  A  E  G  S  ●  Q  I  Q>
P  Y  E  G  V  F  Q  ●  G  V  G  R  R  S  W  G  R  R  F  I  A  D  S>

        80        90       100       110       120       130       140
        *         *         *         *         *         *         *
GTCTAGCGTGGGCTACATAGTGAGTCCTAAGCCAGCCTGAACAGCATAGNCCCAACCTGTTTGGAATAAA
 S  S  V  G  Y  I  V  S  P  K  P  A  W  T  A  ●  X  Q  P  V  W  N  K>
V  ●  R  G  L  H  S  E  S  ●  A  S  L  N  S  I  X  P  T  C  L  E  ●>
S  L  A  W  A  T  ●  W  V  L  S  Q  P  E  Q  H  X  P  N  L  F  G  I  K>

       150       160       170       180       190       200       210
        *         *         *         *         *         *         *
GAAGGAGGAGGGAAAGGAGGAGCAAGAGGAGGAAGAGGAGGAGAAGGAAGAGGAGACAGAAGAGGAGACA
 E  G  G  G  K  G  G  A  R  G  G  R  G  G  E  G  R  G  D  R  R  G  D>
R  R  R  R  E  R  R  S  K  R  R  K  R  R  R  R  K  R  R  Q  K  R  R  Q>
 K  E  E  G  K  E  E  Q  E  E  E  E  E  E  K  E  E  E  T  E  E  E  T>
```

```
       220       230       240       250
        *         *         *         *
AGAGGAGGAGGAGGAGGAAGAGGAGGAGGAGGAGGAAAAGCTT
K  R  R  R  R  R  K  R  R  R  R  R  K  S  F>
 E  E  E  E  E  E  E  E  E  E  E  E  K  L>
R  G  G  G  G  G  R  G  G  G  G  G  K  A  X>
```

Fig. 5 Nucleotide sequence of pλ-7G (flanking genomic DNA sequence only, 253bp) showing the minus strand and amino acid sequences translated in 3 frames. The amino acid residues are represented by the universal one letter code. Stop codons in the amino acid sequences are represented by (●). The cluster of charged amino acid residues in the minus one reading frame is underlined while those in the minus 2 reading frame is shaded. The charged residues are K, R, H (basic) and E, D (acidic). The putative acceptor site, Y-N-Y-A-G/ in the minus 1 reading frame is indicated by the dinucleotide AG, which is boxed.

part of a functional gene, it could not be the first exon and must encode intronic sequences as well. Attempts at identifying a splice acceptor site was made by analysing the surrounding nucleotide sequence. According to Ohshima & Gotoh (1987), the nucleotide frequencies around an acceptor site (consensus: Y-N-Y-A-G/, where Y is a pyrimidine and N represents any nucleotide) typically comprise >70% pyrimidines (C,T, or U) up to positions around -20. However, the authors indicated that the criteria they have used to predict a candidate acceptor sequence may exclude a few real acceptors. Even if a sequence fits into the consensus, it should still be tested experimentally. Only one acceptor site having the consensus sequence Y-N-Y-A-G/ appears to be present in the minus 1 reading frame of *c-7G*. Although this site comprises only 50% pyrimidines up to positions around -20 (Fig. 5), it may still represent a genuine acceptor site. However, more experimental work will need to be done to confirm this.

6.3.5 Attempts to isolate cDNA clones corresponding to c-7G: screening of a C6VL/1 cDNA library and amplification of cDNA from C1-V13D by PCR

Since C6VL/1 was shown to express *c-7G* (Fig. 4), a cDNA library of C6VL/1, donated by I.L. Weissman (Stanford University, CA) was screened with the 7G probe. Of a total of 48,000 cDNA clones screened, none hybridised to the 7G probe, indicating that *c-7G* is not abundantly transcribed in C6VL/1. A second approach attempted was to amplify cDNA from C1-V13D by 3'-RACE (Rapid Amplification of cDNA Ends; Frohman *et al.*, 1988) using the primers 7G and NOT-TT. Three fragments of approximately 700bp, 500bp and 300bp were obtained. All 3 fragments hybridised to the 7G probe (data not shown). They were cycle sequenced with dye terminators using the 7GR primer. No significant homology to the flanking 7G-genomic sequence was observed. The amplified fragments were also compared to sequences in the databases. With the exception of the 500bp fragment, which showed >95% homology with the CTLA-2α and CTLA-2β mRNA, no significant homology (ie. >50%) was seen for the 700bp and 300bp fragments. The CTLA-2 mRNA is expressed in mouse activated T cells and mast cells (Denizot *et al.*, 1989). Full

characterisation of *c-7G* will involve the screening of more clones from a cDNA library constructed from the thymus or C1-V13D.

6.4 Discussion

The 5'LTR sequence in pλ-7G was shown to lack the 43bp and 16bp direct repeats, which has been proposed to confer leukemogenic potential to the moderately leukemogenic pMol502 and V21 BL/VL_3 viral clones (Rassart *et al.*, 1986). However, leukemogenicity has also been attributed to sequence modifications in other viral genes, such as the *gag/pol* or *env* genes (Lenz *et al.*, 1983; Rassart *et al.*, 1986; Tsichlis, 1987; Aziz *et al.*, 1989). A combination of viral determinants, acting in synergy, has been shown to be important in conferring leukemogenicity. For example, the leukemogenic potential of the recombinant MCF 247 viral clone has been mapped to a combined presence of the *env* (gp70 and p15E) and the U3 region of the LTR. Neither gene alone could confer full leukemogenic potential (Holland *et al.*, 1985). The presence of a *Hind*III site at the *gag/pol* region indicated that the 7G provirus is a recombinant virus and could have arisen from recombination between an endogenous ecotropic virus with an internal *Hind*III site in DBA/2j mice and RadLV *env* sequences. The rearrangment of *c-7G* in C1-V13D but not in germline DBA/2j confirmed that it is a newly acquired virus. This acquisition of proviruses by recombination and subsequent re-integration constitute events which are likely to contribute towards the partial transformation of C1-V13D during the period of *in vitro* culture.

There have been very few studies carried out to identify common RadLV integration sites. A recent study by Tremblay *et al.* (1992) demonstrated that in about 5% of tumours induced by the BL/VL3 (clone V-13) RadLV, a provirus is found integrated into a region which was identified as a novel gene, *Vin-1*. Studies made by the same authors also revealed that about 20% and 18% of the tumours show rearrangement in *c-myc* and *Pim-1*, respectively. The *Vin-1* gene has not been sequenced, but was shown to encode a 6kb transcript. There is no evidence to suggest that *c-7G* is equivalent to the *Vin-1* gene. Preliminary analysis of λ clones isolated from the C1-V13D genomic library (Chapter 5) with

^{32}P-labelled *v-myc* as probe has indicated that none of the λ clones carry proviruses integrated into the *c-myc* gene (data not shown).

The integration site of the 7G provirus was unique to C1-V13D and was not observed in the RadLV-induced C6VL/1 and BL/VL3 thymic lymphoma cell lines. Since C1-V13D was derived from a DBA/2j strain mouse and C6VL/1 and BL/VL3 were derived from C57BL/Ka strain mouse, the differences in genetic background could have influenced the integration site. This was observed for Friend virus-induced leukemia, in which integration into the *fim-1* and *fim-2* genes was found to be fairly specific for the B6 X C (F1) mouse strain, while none of the myeloblastic leukemias with ICFW or DBA/2 background showed rearrangments in these two regions (Sola *et al.*, 1986). Since C6VL/1 and BL/VL3 were derived from thymic tumours after infection of mice with RadLV, it is also possible that this integration event is specific to an *in vitro* infected, immature lymphoid cell type like C1-V13D.

Two frames translated from the minus strand of flanking genomic DNA in λ-7G contain long stretches of charged amino acid residues (basic residues: K, R, H; acidic residues: E, D). Extremely long runs of >20 consecutive charged residues, either purely acidic or mixed basic and acidic occur in about one-third of nuclear and cytoplasmic autoantigens. Less than 3% of such runs are found in other mammalian proteins (Brendel *et al.*, 1991). Such clusters of charged residues are also characteristic of eukaryotic regulatory proteins, including transcription and replication factors and heat shock proteins (Brendel & Karlin, 1989). Significant charged clusters are also found in nuclear, but apparently not cytoplasmic proto-oncogene products (Karlin & Brendel, 1990). The long stretch of charged residues $K_2E_2GKE_2QE_6KE_3TE_3$ and $R_4ER_2SKR_2KR_4KR_2QKR_2QE_{12}K$ encoded by the minus 1 and 2 reading frames, respectively, showed >50% homology with some of the proteins reported in the literature. It would be premature at this stage to place undue emphasis on any single homology based on computational comparison, however the homologies do suggest that *c-7G* may code for a protein with important regulatory functions. The possibility that *c-7G* could be part of an untranslated region was not excluded.

Further attempts to isolate the *c-7G* cDNA using PCR amplification gave 3 products. None of these products shared any homology with the genomic nucleotide sequence flanking the LTR. One of the products, however, shared almost 100% homology with the CTLA-2α and β mRNA, which are transcribed in activated T cells and macrophages. The amplified products may not be related to the *c-7G* gene since NOT-TT is not a specific primer and it can anneal to almost all mRNA species. The 7GR primer could also have annealed to other cDNAs with nearly similar homology and allowed for amplification of these cDNAs.

SUMMARY

A single 5' LTR-host junction subcloned as pλ-7G was analysed in detail. Comparison of the flanking genomic DNA with sequences available in the GENBANK and EMBL nucleic acid databases failed to detect any significant homology. Southern analysis however showed that the *c-7G* gene is a unique sequence and produces different RFLP patterns in the strains studied. It is also specifically rearranged in C1-V13D but not in other RadLV-induced or other tumour cell lines. It appears to be expressed by cells of lymphoid origin and not by adult liver cells. Preliminary analysis indicated that it is possible that a part of the protein encoded by *c-7G* contains a stretch of charged amino acid residues (>20 a.a.) in the minus reading frame. The presence of long stretches of charged amino acid residues has been reported to be associated with transcription factors and nuclear proto-oncogenes. *c-7G* may code for a protein with similar functions, which would be important in regulation of growth and transformation of an early lymphoid cell type like C1-V13D.

CHAPTER 7

General Discussion

7.1 A model for study

The ability of type C murine retroviruses to induce leukemia is influenced to a great extent by the genetics of the host (Rowe, 1972). The development of leukemia usually occurs after a long period of time. To circumvent this, an established, partially transformed retrovirus-infected cell line was investigated as a possible model to study the oncogenic process in T cell leukemia. RadLV, a type C murine retrovirus, was used because it provides some interesting aspects for study. For example, it has a preference for immature cells in the thymus as targets for infection, and induces leukemia in mice after a lengthy process of clonal selection in the thymus. This allows the study of thymic involvement in T cell leukemia. In addition, the RadLVs do not carry any cell-derived sequences and elicit tumours by disrupting host cellular genes. They are therefore potentially useful for uncovering novel proto-oncogenes involved in the tumorigenic process in early T cell progenitors.

The RadLV-infected cell line, C1-V13D has a unique lymphoid precursor phenotype (O'Neill, 1992). It was used in this study to investigate genetic changes occurring after initial infection which could be associated with disease progression. These include retroviral recombination and/or integration events, any differentiative changes due to the thymic microenvironment and the disruption of cellular genes by retroviral integration. Although C1-V13D could grow as a tumour in syngeneic DBA/2j mice via most routes, it was unable to grow subcutaneously in unirradiated DBA/2j mice. Also, its ability to grow as a tumour in allogeneic mice was restricted by strain and route of inoculation. It is therefore a partially transformed cell line. C1-V13D has demonstrated a specific capacity to grow in the thymus and to become

selectively more tumorigenic after serial intrathymic passages. This observation provided the basis for evaluating favourably the usefulness of C1-V13D as a potential biological tool in the dissection of oncogenic event(s) occurring intrathymically during disease progression.

7.2 The oncogenic process

7.2.1 A multitude of events

Many naturally occurring tumours, including those which are virally-induced, evolve by step-wise genetic changes as part of the oncogenic process. For example, although integration of the avian lymphoid leukosis virus (ALV) into the *c-myc* gene was found in the majority of diseased birds, not all of these birds showed evidence of increased *c-myc* expression. Further analysis showed that DNA from ALV-induced lymphomas could transform recipient mouse cells, but the transforming DNA contained no *c-myc* sequences (Hayward *et al.*, 1981). It was suggested that integration into *c-myc* may simply be an initiating event, serving to render the cells susceptible to further oncogenic changes (Wyke, 1981). A similar mechanism has also been proposed for Burkitt's lymphoma in humans, in which the Epstein-Barr virus selectively infects B cells and renders them susceptible to additional neoplastic events. These results demonstrate that more than one mutational event is required to activate the full oncogenic potential of a single proto-oncogene. Cooperation between two oncogenes, where one is associated with a later stage of disease development by further provirus insertion, is another example (Section 1.5.3). A sequential activation of one oncogene after another was considered necessary to induce a fully malignant state.

In this study, the increasing tumorigenic potential exhibited by C1-V13D cells after each passage through the thymus reflected changes indicative of stepwise oncogenic progression. While the involvement of the thymus in T cell leukemogenesis has been well established (reviewed in 1.5.1), its role as a unique microenvironment in regulating the growth of pre-leukemic or leukemic cells is less well

understood. This study has focussed on genetic and phenotypic changes occurring in C1-V13D cells during oncogenic progression as a result of replication and residence within the thymus. Based on evidence presented that cellular genes could be disrupted by retroviral integration, resulting in partial transformation or maturation arrest of cells, the characterisation of some of these genes in the vicinity of retroviral integration in C1-V13D was also initiated.

7.2.2 The right microenvironment?

A wealth of literature supports the view that immature cells are 'selectively' targetted for initial neoplastic transformation. The susceptibility of immature cells has been demonstrated in leukemia (reviewed in Section 1.4) and also in other types of cancers like teratocarcinomas and colonic tumours (Jacob, 1980; Barnhill *et al.*, 1993). In the case of hematopoietic cancers, immature target cells exhibit phenotypes indicative of multipotential or bipotential cells. A consequence of this is that transformed cells cannot be definitively assigned to any particular cell lineage (reviewed in Section 1.4). Based on the concept of 'lineage promiscuity' (Greaves *et al.*, 1986a), it could be assumed that the choice of commitment of these bipotential or multipotential cells to a particular lineage is intrinsically flexible. Theoretically, these transformed immature cells could give rise to a wide spectrum of leukemic diseases, depending on which growth factors are present and the microenvironment in which they develop (Diamandopoulous *et al.*, 1979). This has been demonstrated in a number of functional and phenotypic analyses which showed that growth factors were capable of dictating the lineage commitment of immature T-ALL (acute T-lymphocytic leukemia) cells (Kurtzberg *et al.*, 1989; O'Conner *et al.*, 1990). In another study, a subpopulation of uncommitted cells present in a mature T cell leukemia was shown to undergo multi-lineage differentiation depending on the growth factors supplied (Griesinger *et al.*, 1989). Engraftment of chronic myeloid leukemic cells into the thymus was reported to result in the differentiation of donor leukemic cells into T lymphocytes (Gishizky *et al.*, 1993). Taken together, these data show that well differentiated leukemia can change phenotype when exposed to a different microenvironment. This is indicative of the

existence of an immature leukemic cell pool which is capable of giving rise to cells of different lineages.

In the model described here using the spleen-derived, RadLV-infected C1-V13D cell line, two features highlight the importance of the microenvironment: a) the thymus was the only organ with little restriction to replication of C1-V13D in allogeneic mice, and b) an increased number of viral particles (Chapter 4) was observed in C1-V13D progeny cell lines reisolated after intrathymic passage, indicative of viral replication and productive infection. The ability of the thymus to support proliferation of leukemic cells like C1-V13D and to select for cell clones with increased tumorigenicity highlights the uniqueness of the thymic microenvironment. The capacity of C1-V13D to proliferate in the thymus and to acquire T cell surface markers suggested that it could be a precursor T cell. This is consistent with earlier evidence that RadLV preferentially infects immature T cell precursors, and that viral replication requires interaction of these cells with thymic cellular elements and factors present in the thymic microenvironment (Goffinet *et al.*, 1983; Boniver *et al.*, 1989). The presence of recombinant viruses (eg. MCF viruses) in C1-V13D (Chapter 5) is also consistent with other evidence that MCF viruses also selectively replicate in rapidly dividing immature thymocytes present in the thymic cortex (O'Donnell *et al.*, 1984). Increased viral replication may directly or indirectly alter cell growth and thus contribute to the oncogenic process. This has been documented for the MoMuLV (Tsichlis *et al.*, 1987)

7.2.3 The preleukemic or leukemic cell

There has been considerable evidence presented to suggest that the leukemogenic process is linked to the cell's normal differentiation program (reviewed in Section 1.5.2). A cell's susceptibility to leukemic transformation by retroviruses was shown to be dependent on its differentiative state, thereby providing an example of how the developmental state of a cell could be exploited by leukemogenic agents. This has been documented in the case of type C retrovirus-induced leukemogenesis (reviewed by Kaplan, 1961), where stem cells or lymphoid progenitors were shown to be most vulnerable to

infection by these leukemogenic viruses. In addition, normal differentiation events, such as the rearrangement of immunoglobulin (Ig) and T cell receptor (TCR) genes have been shown to contribute to malignancies in B and T cells, respectively. A frequent occurrence is the translocation of a growth regulating gene into a highly fragile region such as a rearranging Ig or TCR gene locus. This results in transcriptional dysregulation of the growth regulating gene and unregulated cell proliferation (Yunis, 1983; Croce *et al.*, 1985). These examples also demonstrated clearly how the normal process of cell differentiation could effect leukemic transformation.

The capacity of leukemic lines to respond to physiological inducers and to differentiate *in vitro* has been demonstrated in a number of independent studies (Friend *et al.*, 1971; Sachs, 1978; Paige *et al.*, 1978; Collins *et al.*, 1978; Lazo *et al.*, 1990; Hibi *et al.*, 1993). A similar capacity to differentiate *in vivo* was shown to be very much dependent on the maturation status of the leukemic cells and the microenvironment in which they replicate (Green *et al.*, 1985; Lazo *et al.*, 1990; Gresser *et al.*, 1991; Gishizky *et al.*, 1993). It has also been observed in T cell leukemia that tumour progression is associated with more mature T cell phenotypes (Lieberman *et al.*, 1979; O'Donnell *et al.*, 1984; Yefenof *et al.*, 1991). This has also been documented for other types of leukemia (Jensen *et al.*, 1992). C1-V13D cells were shown to differentiate and express T cell surface markers. They also acquired increased tumorigenic potential while replicating in the thymus. These changes probably reflect maturation events associated with oncogenic progression and manifestation of the disease.

Analysis of the effect of oncogene expression on cell differentiation has been limited by the availability of good experimental systems. *In vitro* cultured cells have been used widely for this purpose. Key selection criteria appeared to be a) purity of cell type, b) ability to divide and c) ability to retain expression of normal differentiated cell phenotype (reviewed by Boettiger, 1989). Phenotypic and morphological analyses of C1-V13D cells have already indicated their lymphoid origin (Chapter 2). The inability of sorted, Thy-1 positive C1-V13D cells to retain a differentiated T cell phenotype in tissue culture did not indicate a clear

association between differentiation and oncogenic progression in this study. However, genetic events associated with early differentiation of C1-V13D cells may have occurred and remained unrecognised in the absence of available phenotypic markers. The evidence presented in Chapter 3 is consistent with other evidence indicating that differentiation is intrinsic to the leukemogenic process, and supports the hypothesis that maturation within the thymic microenvironment is important for oncogenic progression in C1-V13D cells.

7.2.4 The role of the virus

In some systems, further retroviral integration and recombinant events have been shown to contribute to the leukemogenic process (reviewed in Section 1.5.3). The possibility of these events contributing to oncogenic progression in C1-V13D cells was investigated. However, the data presented in Chapter 4 showed no evidence of further retroviral events involving RadLV or RadLV-like *env* gene sequences. Newly integrated viruses, representing recombinants involving the *pol* gene were observed in some primary clones but *in vivo* tests for increased tumorigenicity associated with the generation of new recombinant viruses suggested that the retroviral events detected were probably random or incidental (Chapter 4). This analysis did not address the significance of multiple retroviral integration events which had occurred during *in vitro* culture of C1-V13D cells following the initial infection. Subcloning of genomic DNA in the vicinity of retroviral integration was therefore carried out to isolate and characterise genes which may be responsible for the partial transformation and maturation arrest of C1-V13D cells *in vitro*. Since certain recombinant virus structures have been identified and proposed to be important for clonal selection during tumour development (van der Putten, 1981), the analysis of recombinant viruses which have evolved during transformation of C1-V13D *in vitro* was also attempted.

Restriction enzyme analysis of viral genomes has revealed the presence of both recombinant and defective viruses. This was evident by the presence of a *Hind*III site in several cloned proviruses. This site is not present in the original RadLV/V13 clone (Rassart *et al.*,

1986) used to infect and immortalise spleen cells, from which C1-V13D was derived (O'Neill, 1992). The consistent presence of a *Hind*III site at the *gag/pol* region in all full length viruses cloned suggested that this virus structure may play a role in cell transformation *in vitro*.

7.2.5 The role of proto-oncogenes and other genetic elements

Simple repeat sequences, once considered to be meaningless or "junk" DNA have become increasingly important in disease association (Kunkel, 1993; Thibodeau *et al.*, 1993; Mironov *et al.*, 1994). There is increasing evidence to indicate the involvement of LINE and SINE repeat elements in many cancers. These repeats are also termed mobile genetic elements because of their ability to retrotranspose. These elements have now been shown to have regulatory roles and may encode proteins (Section 5.4). The transposition of these elements into single copy loci could have profound effects on gene activity (Economou-Pachnis *et al.*, 1985; Katzir *et al.*, 1985; Arman *et al.*, 1986; Morse *et al.*, 1988).

Translocations involving fragile sites or breakpoint cluster regions in specific chromosomes are common in T and B cell leukemias. Sequence analysis of the breakpoint cluster region in the ALL-1 gene in acute leukemia has revealed a high density of *Alu* repeats, which are equivalent to the rodent B1 repeats (Weiner *et al.*, 1986). Seventy percent of these breaks were found to occur in the region delineated by exons 6 and 7, which is composed mainly of *Alu* sequences. It was proposed that the high density of *Alu* repeats could make the breakpoint cluster region more prone to recombination (Gu *et al.*, 1994).

So far, no studies have linked repeat elements with retroviral integration sites. A LINE sequence, inserted 6kb from the region of MoMuLV integration (*Mlvi*-2), was proposed to be a genetic change required for neoplasia (Economou-Pachnis *et al.*, 1985). The presence of B1 and B2 repeats in the vicinity of all full length viruses cloned from C1-V13D (Chapter 5) suggests a possible association between repeat elements and viral integration sites. Several interesting

possibilities exist: a) a genomic region containing repeats is a 'hot spot' for events such as recombination and integration, b) repeats and retroviruses could cooperate with or antagonise each other, leading to disruption of gene function, or c) the presence of one in the vicinity of the other could promote additional recombination or enhanced retropositioning, resulting in further genetic instablity. Another interesting possibility is that these repeat elements may themselves be a gene, or part of a gene with specific function, which can be activated by retrovirus insertion. The Set 1 and 2 transcription units, which contain dispersed repetitive elements, are examples. These units have been shown to be activated in SV40, Abelson MuLV and Rous Sarcoma virus-transformed cells (Scott *et al.*, 1983). Activation of genes of this type by retroviral insertion may contribute directly or indirectly to the transformed phenotype. It is possible that retroviral integrations in the vicinity of repeat elements in C1-V13D may not be completely random and may have a role in the transformation of C1-V13D. This possibility was considered in view of the biological role of repeats as regulators, their documented involvement in cancers as genetically unstable mobile elements and as possible hotspots for recombination and retroviral integration.

The identification of proto-oncogenes that play important roles in tumour development has led to a better understanding of the molecular events underlying the oncogenic process. Characterisation of common integration sites in MoMuLV-induced lymphoma has led to the discovery of genes associated with tumour initiation and progression (Tsichlis *et al.*, 1990; Bear *et al.*, 1989; Breuer *et al.*, 1989). Analysis of the LTR-host junction has been used as a procedure for isolating novel genes associated with tumour development. In this study, the 5'LTR-host junction, subcloned from λ-7 and designated as pλ-7G, was fully sequenced. It was possible that it could be part of an untranslated region. However, translation of the nucleotide sequences proximal to the 5'LTR in pλ-7G has revealed that it could also code for an interesting stretch of charged amino acid residues. Similar stretches of charged residues have been reported in association with eukaryotic regulatory proteins, nuclear proto-oncogene products and many G protein-coupled receptors (Karlin & Brendel, 1990). The disruption of genes coding for these

proteins by retroviral integration could result in disruption of normal cellular functions.

7.3 Strategies for future investigations

The results of this study suggest an association between differentiation and increased tumorigenic potential in C1-V13D following replication of cells in the thymus. However, the possibilty exists that genetic changes associated with early differentiation are not reflected in the acquisition of stable phenotypic markers. Another approach to identifying genetic changes during oncogenic progression in C1-V13D would be to employ karyotypic analysis to check for cytogenetic lesions in the primary, secondary and tertiary clones reisolated after first, second and third passage through the thymus. Any cytogenetic lesions associated with specific chromosomes could be analysed for the presence of genes associated with cell differentiation. Recent advances in the mapping of the mouse genome should facilitate the linking of these genes to specific chromosomes (Copeland *et al.*, 1993).

Further characterisation of the subcloned LTR-host fragments, including *c-7G* could provide interesting information on novel proto-oncogenes and the regulation of early lymphoid cell proliferation. A preliminary attempt to isolate the cDNA corresponding to *c-7G* from a cDNA library of the C6VL/1 T cell lymphoma line was unsuccessful, suggesting that *c-7G* mRNA is a transcript with low copy number. Further work is needed to subclone the full length *c-7G* cDNA from a thymic cDNA library or from a cDNA library constructed from C1-V13D cells. The intervening DNA regions in subcloned $cDNA^+$ fragments, which do not carry any repeat sequences and which have no significant homology to sequences in the GENBANK and EMBL nucleic acid databases could be investigated further. Computational analysis has indicated that some of them may be parts of uncharacterised gene sequences since they have high coding probabilities. These sequences could be used as probes to analyse Northern blots in order to determine if they are specifically transcribed in C1-V13D cells. Any transcribed regions found

could be used to screen a cDNA library of the appropriate tissue or cell line and positive cDNA clones could then be isolated, sequenced and characterised. Putative regulatory sequences (enhancers or promoters) could be assessed for their function by subcloning into an appropriate expression vector, for example, the pCAT reporter plasmids (Promega, USA), where their ability to regulate the expression of a reporter gene can be determined.

7.4 Summary and general conclusions

This study presents an attempt to elucidate some of the mechanisms involved in T cell leukemogenesis using a partially transformed cell line, C1-V13D. This cell line was derived by *in vitro* infection of primary spleen cells with RadLV, a non-oncogene bearing murine type C retrovirus. The data obtained is consistent with published data on the involvement of the thymus in supporting proliferation of leukemic cells and retrovirus replication. Oncogenic progression was seen to occur in C1-V13D cells after intrathymic replication. Analysis of phenotypic changes revealed that C1-V13D cells differentiated under the influence of the thymic microenvironment, consistent with progression towards a more mature phenotype during tumour progression in T cell leukemia. No further retroviral integration events were observed involving the original RadLV virus, as indicated by Southern analysis using the specific RadLV gp70 *env* probe. However, recombinant and defective viruses were detected and several of these were subsequently subcloned and characterised. These viruses were present in the original C1-V13D cell line and probably arose during the period of *in vitro* culture. These retroviral recombination and reintegration events could have resulted in the partial transformation and maturation arrest of C1-V13D cells. Finally, subcloning and analysis of genomic sequences in the vicinity of retroviral integration from a C1-V13D genomic library revealed the consistent presence of repeat elements of the B1 and B2 family. It is possible that these elements represent 'hot spots' for retroviral integration. Destabilisation of these mobile genetic elements or disruption of their normal regulatory role via retroviral integration could be important in oncogenesis. Sequence analysis of a transcribed

genomic fragment adjacent to the 5'LTR in the pλ-7G genomic clone has revealed the possibility that the particular gene involved could encode a stretch of charged amino acid residues. Stretches of such charged residues are found in proteins important in regulation of cell functions. Subcloning and characterisation of its full length cDNA will be the next step towards understanding its involvement in the transformation and maturation arrest of C1-V13D cells.

APPENDICES

Appendix 1 Nucleotide sequence of pλ-1BB (1.187kb)

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   1 GCAGCATAAT CAGAGTCAGG CTACCTTACC TGCTTAAAAG CAAATAGCAG CCGGGCGTGG
  61 TGGCGCACGC TTTAATCCTG GCACTTGGGA GGCAGAGGCA GGCAGATTTC TGAGTTCGAG
 121 GCCAGCCTGG TCTACAAAGT GAGTTCCAGG ATAGCCAGGG CTATACAGGG AAACCCTGTC
 181 TCGAAAAACA AACAAACAAC AACAAAAAAA AGAGCAAACA GCAACCAGGG CCGAATAAGT
 241 GCCACTGAAT TACTCTCTCC TCCCAGTATA AAAAGAGGTT GACGGAGCCG GGCGTGGTGG
 301 CGCACGACTT TAATCCCGGC ACTTGAGAGG CAGAGGCAGG TGGATGGATT TCTGAGTTGG
 361 AGTCCAGCCT GGTTTCCAAA GCGAATTCCA GGATTAGTCA GGACTACATG GAGAAATCTT
 421 TGTCTTCACA AAACAAAAGG GAGAAGAAGG GAGTTTGACT GCGGCAGATG GTCACAGTTG
 481 CGTTTGCTCT TTTCTTCTCT CCTCCTCCTC TTCCAGGAGA TGGCAAGCCC ATGAGCAACG
 541 TGGCTCCCTG TCTGGGGCTT CTGTTATAAA GCTGGCCCAA AGGAGNATGA TCTTCCCTAA
 601 CAGCTCCCTT CAAATGTCAC CGTTTCCTAA GCTCGGTGTT ATCCTGGGTT AACCTGGTTC
 661 TGTCTGGGAC CTTCCCTTAC AGTAGGAGAC GGGGTGGCTG CCAGCCCCTG GCATAGAGGG
 721 TATCTGTGCT CAGGAAAGGT AAGCTCATAC TAAGATGCAT AGGGCGCTTC ATACTGATCG
 781 ATCCCTAGGA CCAATAGGAG CCATCTGGAC CTAGCAAACT TTCCCCTACC CCTAACAAAG
 841 AAGCTGGTTC CCACCCAACA CCAGGGGCTC CCTCACTTCA CAGTCCAGTC ACAAGGAAAG
 901 GACAGACAGG GTGGTGGTGA CAGTGGCCCA GAAATGACTT TAGCCAGGGC TAGGTCCTTG
 961 TTCCTGCTGT CCCTGCAATT CTCATTTGGC TTGTCTGAGC CTCAGTTTCT TCATTCATTA
1021 AAGCCGGCAT GAGGCACCCA CCTCCTTCAG GCCAAAGGAA CAAAAAATGA CTGACAAGTG
1081 CCGGGCCCTG GGAGGAGGGG CTCCCAGCAA TGGAGCACCT GGGCAGGATT CCTAGAGGGA
1141 GTGGGCTACT GTATGTATCC TAAACTTGGT AAGGACGCCG CTCCTCTG
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Shaded region represents repeat sequence. The poly(A) tract is underlined.

Appendix 2 Nucleotide sequence of pλ-7K (2.610kb)

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   1 AAGCTTGAAA TGTTCTCTTA AAAAGTGGCT TAAGGGCTGG AGAGATGGCT CAGCAGGTAA
  61 GAGCACTCGA CTGCNCTTCC GAAGTCCTGA GTTCAAATCC CAGCAACCAC ATGGTGGCTC
 121 ACAACCATCC GTAATGAGAT CTGGTGCCCT CTTCTGGTGT GTCTGAAGAC AGCTACAGTG
 181 TACTTACATA TAATAAATAA ATCTTTTAAA AAAAAAAGGT GGCTTAATGG TACAGTGCTC
 241 ACCTATCTTA TTTGTTTGTT TNGTTTTCCT GTATAGCCCT GGCTGTCCTG GAACTCACTT
 301 TGTAGACTAG GCTGGCCTCG ATCGAACTCA GAAATCTTCC TGCCTCTGCC ACCCGAGTGC
 361 TGGCATTAAA GGCGTGCGCC ACCACGCCCA GCTGATGCTA GTATTTTATG AAGGAAGAAA
 421 AAAGAAACCA ATAAATGGAT ATGCATGCAT GTACTTCTAT TCTTGGAGCC CAAAGGAGAG
 481 TAAGACAGGG AGATTCCTAA CTCAAGGCCA GCATGAGCAA CATAGTGACA CGTGTCTTTG
 541 AATCCCAGCT CTTGGAGGGC AGAGGCAGAT GGACCTCTGT AAGTTTAAGG CTAGCCTGGT
 601 CTACAGAATT AGGACAGCCA GGGCTACACA GAAAAACATT GCCTCAGAAA AACAGAATAA
 661 AAGAAAAAAT AAACAAAAAC AAAACAATAA TGCAGAGGTG AGGAGATATT TGTGAGTCCA
 721 AGGCCAGCCT GGTCTACACC TGGAATTCTA GGCCAGCCAG GGCTACAAAG TGAGACTCTG
 781 TCTCAAAAAA ACCAACAAAA CAGAAAGCCA CTTCAAAAGC GTCCAAACAC TAAAAACCGG
 841 GACAAACGAG GTTTTGTTTT ATTTTTCACA TGAAGAAGCA GCAAGGAAGA AAAATAAATA
 901 AATAAATAGT TGGTTGTGTG GGTTTGAAAA GCCTATGGGA AAATAATTAA CTAGTGGTTG
 961 GCACCCTCCT TTGAACAATG GCCCAGTCTC CAGATTGACG CACACAGTTT GGGGTCCAGC
1021 TATTGAAGCA GTCACTAGGG GTGGCTCTCA GTACTCTCCA CACTGTAGCT CCTTGAGACT
1081 GGAGTCAGCC GAGCACCCCA ATAGCCTTGG GAGGGTTGTG GCGAGTCGCA CTGTCCTGGG
1141 TTCTGATTCT CTGAGAAATT GCAAGGGCTT AGCAGGATTG TGAATATTCT AATTAGGCAT
1201 GTGTAATTTG ATTTTGGAGC GGTGGAACTT GTGAGGTCTT TTGAACCTAC ACAGGAGAAA
1261 AAACAACTTA AGCCTTTGAA AATGAATGGA CATATCAGTG GATTATGATG CACACAAGTC
1321 CCTCCAGCTT TGAGCCCTGC TCTGGGATAT ATCGGATGCC CAATAAATTA ATAAAGTTGT
1381 TGATTTGAGT GGGAGTCCGT TGGACTCCAA AAAGAGGATG GGTTTCTCTG TCTTTCCACA
1441 AAGCCCAACT CTTTGATCAC ATCAAGGCCT CTGGCCACTG CTCTGCCTAC AGTAGTTAAC
1501 TCCATTCACT CAGTCTTTCA CCATTCTTTG GAAAAGAATA CTCCTCTTTC CTTCCTTCTC
1561 CCCCACCCCT TCTTTCCTTC TCTTTTTGAT GCAGGNTCCC CCTATGCAAC CCTGGCTGGC
1621 ATGAACTGAC TGTGTAGACC AAGCTGCCCT TGAACTCACA GAGATCTGCC TGCCTCTGTC
1681 CCCTGGGTGC TGGGACTAAA GGTGTGTGCC CAGCCATGCC CAGCCACTTT TCTTTTTTAG
1741 GATCTAACCC CTTCTGATCC AAGTGTGAAG TGCCACTTCC ACTCTTAGTT CCAATCACCT
1801 CCCCTTGTCA CGTTTGCAGA GAGACCTGGG CCAGACTGCC CAAACCCAAT CTGCTTCTCT
1861 GCTCCTCCTC CTTCTATTAG ACTCTCTCCC TGTCTCTCCC TCCCTTCTCC TCTTATTCTG
1921 CATAGTGTGT GTGTGTGTGT CTGTGTGTAT ACAAAAGGGT TATTCACTGA ACCCGAAGCA
1981 GCTCACCTGT TAGGCTAGAC TAACCAGCTT GTGAACCCCC AGAGCCTCCT TGTGCCTGCC
2041 TCCCCCAGTG ATGAGATTTC ACATGTACTC TACAACCAGT TTTTTTTTTT TCTTCCCCAT
2101 GAGTCCCGTG ATCTGAACAC AGACAGGTCT TCGTGTTACC AGGGAGCTTG GGGCCTCCTT
2161 TCATAGGTTC TTAGTCCCCT TGATGCAAAT TTTTAAAAAG AATTTGGAAG GACTTAAGGA
2221 CCAGCATGTA GAGTCCAGGA GAGTTTGGAG GAGAGGCAGG CTGCAAAGCT CGGCAGCTGC
2281 CAGGAGGAAG TAGGTGGAAA GGGAGAGGAG AGAAAGCCAC ATCTTTAAAA TTACAGTCCA
2341 AGAAGGCACA GGCTCCGAGA TGGAGGCAAG CAAACAGCTG TGTGGCAGAA GTGGGGTCTT
2401 CAGAAAAAGA GTGAAAATGT CCAGAGGGTC ACGAAAGGAG ATACGTACTC AGAAAGGGAG
2461 AGGAGCCGGG TGCAGTGGTG CTCACCTTCT GTCCGCAGGA GGCAGGACAG GTTCTCTATG
2521 ATTTTAAAGC CAACCTGGTC TGCATAATTG GATTCCAAGC CAGACTTACA TTGTAAGACC
2581 CCGTGACCTT GGATTTCTTG ACATTCTTCG
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Shaded regions represent repeat sequences. The poly(A) tracts are underlined.

Appendix 3 Nucleotide sequence of pλ-13AB (2.48kb)

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   1 AAGCTTTAAG GTTAAATGTA ACTCTATTTA AAGTAGGTAT CAGAGCTGGA GGAGCCAAGA
  61 GTTGAGTCGG CAGCAGTTAG CTTTTAAGAG GACCTTGGTT CAGTTCCCCA ACGTGGCACT
 121 CACACTGGTC TGACCCCACC TCCAGGGGAT CACACACCTT CTTCCAGCCC CTATACCATT
 181 TGCATGTGCA CACGTACATG TACAGGTGTA CACACACACA CACACACACA AAGAGCGGAA
 241 CATGGACATG TAAGTATTTA AGTAGTTCCC ATGAAACTCA TACAGGAAGA ATGTCCACTG
 301 GGGAAGCTCT TTCGGCCCTC GTTCACCACA TCCTCGTCCC CCGTGCACGT CAGATCAGAA
 361 TGGGCCAGCC CATCAGGGAA GCAGCGGTAG AAGAAGTCTG GGCGGGGCCT GGAAGAAATA
 421 TAATTTACTC TCTCAGGAAG TATTTTTCAG TAATAGTAGA TGTCAATAAT CTGTTGACCA
 481 CTAATGTCAT AGGCAAAATA ATGTAATGGG GTGAGATTAG TGTTTATACT GAGAAAAAGG
 541 CCTTAGCCAC GCGGCCAATG TTCAAGAAAG TCTCTAGCAT ATGATTGTAT CTTAGCACAG
 601 AGCAGCTAGT CATACTGCAG CTTTAGGTAC ATAAAGGTGG TCGCTGTGGG AAATACTCAG
 661 GACAGGACGC AGACCAGGTG GTACACTTGG AACAGGCAGA GGGCAGGGAG AGGGAGTTCT
 721 GGTGAGAAAG GAGAGACTAG TCTGAGAATC CGGGTAAACA CTGCTTGAGA ATGTGGTGGT
 781 GTGTGTGTGG GGGGCGCTCA GGGAGGTTTG AGTGCCAAGG CTTCTCACTT ACCTGCCCAC
 841 TATCAGTTTG ATTATGTTGG TAAAGACACC ATTCAGAGCT AGGGCAAGGC TGGCAGCTGG
 901 AAAGCAAAGA AGCCGTTAGT GCCATCTGCT TGGAGAAGAG TAAGCCACCA CCTCATGAGT
 961 TAGAGCCTTC ATTGCTTTGA GATAGAACCC AACCAGAATA AAAACCCAAA CGGCCTAGGG
1021 GCAGAGAAGT GGAGCACTGA GGAACACCTA TGTGGCACCT GTGTTAATAG GGAACACCTG
1081 TGTGGTGTCT GTGTTAATAA GGAACACCTG GCCGGGCGTG GTGGCACACG CCTTTAATCC
1141 CAGCACTTGG GAGGCAGAGG CAGGTGGATT TCTGAGTTCA AGGCCAGCCT GGTCTACAAA
1201 GTGAGTTCCA GGACAGACAG GGCTACACAG AGAAACCCTG TCTCGAAAAA ACAAACAAAC
1261 AAAAAGGGAC ACCTGTGTGG CGCCTGTGTT ATTAAGCACA TTCTCATCAG TACTGCTTTT
1321 TGGTCTCATG GGGTAAAAAG ACATCTTTAG GGGTACAGAG TACTGAACCC CATAGCTACT
1381 TGCCAACACT AAGACAGGAA CAACTGGAAC AAGGGCGACT TACCGAGGCA GGCTTGCTTG
1441 CTGTCGGTAG CGTCACTTTC CTTAGAAACT TGGCGAGGAA GTTCAGGGAC AGTGGAGTGA
1501 GAAAGGCAAT GACCTGAAGG TAAAACACAG GGTCGGTAAA ACCAAGTCCC TCCTGGCCTA
1561 AAGCACCAGC TGCCGCGCAC TAACCAAGAT CGGGGCTNAG ATTGCCCACC AGACGCCTCG
1621 CCCTAGCCCT GGAACTCAAG GCAGGGGAGG AGTCCCTGCC AGCGGAGGAG GGCGGGAGGG
1681 CAGCTCACGC ACAAACATGC GGCCGGTGGG GAAGTATTCC GCCTCCACGT ACGGGTTCCT
1741 GTAGAGCCAC AGCTCCTCGG GCTGGATCCG CCGCTGGAAG GGAGGGAGCA GCTCGGTCAC
1801 CCTGGGGAGG AAACGGGGAA GATTGACAGG CTACGTCGGC GGAAGAACCT GCCGCCCCGA
1861 GCCGCTTAGG AAGTGCGAGC GCCCAGGGAC GCCTGGAAGC GTTGACAGAT CCTTACAGGA
1921 AAGCCACAAA GAGCAGGACC CGCACGCCCA GCTCGGCTCC CAGGGNCGCC GTCCCCATCC
1981 CGCCATACGT GCGGTTCAGA CGCTGACGTG GCCCCGGCGC GCCGTGGTTC CGCCCGCCCG
2041 GTGTCCAGCG CCACCTAGCG AACTCCGCGG GGACTGACTA CCAGCGGGGC CTCCGGGCTA
2101 CAGGAAATTC AGAGCTCATT TGCTGTGTTA TGAGTACCAC CTAATAACAG ATCACCTTTG
2161 GGTCTCAAAG CAATGTGTCC TTCACCAACC ACCCAGATCA GCTTATAGGA CAAGAAAAGG
2221 AACGTCATTC TCCAGAGATG CTTCCTCTAC AAAAGCAAAC CAGAAAGCCC ACTTTAAAGA
2281 AACTATGTCT TTGGAGCATT TCGAAGNGAA AAAACTGGGC TGAGGGTGAG TGGTTGTATT
2341 TTTGTGTCTC ATTTCCCAGT AAACTGTACC ATGAAATTAC AGGTAAAGAA CCAGCCCATA
2401 TTCTTAAGTA GACCATACAG GACAATTTAT TAAAGTCAAC CTCAAGCAAG ACCTGAAATC
2461 AATGGAATGC CATTAAAAGC
```

Shaded region represents repeat sequences. The $(CA)_n$ repeat sequence is boxed.
The poly(A) tract is underlined.

Appendix 4 Nucleotide sequence of pλ-17D (3.848kb)

```
   1 AGCTTAGATGTGGTGCTGGGGCAGGGGAGAGTGGGCAAGGAGAGCCAGGTGTGCCCAGAGGGTGGCAGGC
  71 CAGGTTGTGTTCAGACTTGTACATCACTGACTAGGCTCTGTTTACAGACCAGTGTCCTCATCTGTACACC
 141 ACAGGAGCTGGGGTGGAGAAGGAGGATGGAGCCCATCCAAGAGGATGAGGGTGGGGTGGTGGTGGGGTTG
 211 GGGCCTATTCCAGAGAAGGTAATTATAGAAGCAGTTTACCTGGCTGACCAATGGAGCCCCTGAAAGCTCT
 281 GTAGAGTCAGCACCATACCAGGAAGCATTGGGAAGGGAGGTGGGGTGGGGTAGGAGAGTAAGATTTGAAC
 351 CCAGGGCCTAATACAGGCCAGATAAGTGTTCTACCACTGAGTCACATCTCTAGCTTCCTAAATTTCCCCA
 421 AAAGAAACAAAGCAAAGCAAAACAAAAGCCAGGCTGGGAGTGTAGGCCAGCAACAATGTTCTTCAATAGC
 491 ACGCATGAGGCTCTGCTTGGTTCATCCCTAGGACAGCTCACATCCACTAAGTACTTGCCAAGTCCCACAG
 561 CATCCTAACCTTTTTACATCTATCTGTCAGTATACAGAGTTCTGTGCAACCCAGTATATTCTGAAGGCAA
 631 ACCATCAGCTTCAAATCCAGCCCCTACCACTTCCCAGCTGATGTGACTTTTTGTTCAATTTACTGTGTAN
 701 CTTAGAANCTCAGTTTTCTCCTCTGCAAAGTTGAGGTAACAAAGCACTAACACATGGGGCTGTGTGAGGA
 771 CCGAATGGAATCAGACTGCTTCCCTCAACTATGGAGTCTTATCAGAAGTCATCAGCTGGGTGTGGCACCT
 841 GGCATGATTCTCACACAGCCTAAAATTGGAAGCCCTGACCTCATTTTATAGATGAGAGCGAGTTGAGTTC
 911 TGAGAGTAAAGAGACTTGTCAAGTCATACGGCTAGAAACTTAACATGGCTGAGCCTTAACTCTGGGGTTT
 981 AAAGTTTTTGAGCTCTTGCTCAGAAGTCTGGGACCAGACTGGAGAGATGGCTCCACAGTTAAGAGCACTG
1051 ACTGCTCTTTGAGAGTTCCTGATTTCAATTCCCAGCAACCACAAGGTGGCTCACAACCATCTGTAATGGG
1121 ATCTGATGCCCTCTTCTGGAGTGTCTTCAGACAGCAACAGTGGACTCCTAGAAGCATACTCTCGACCCTA
1191 ATTCTTCTGAGTCTTAGGCAGGATGCTCTTTAGCTCCAAGCCAGCCTGGACTACAGAGCAAGGTCCTAAT
1261 TTAATAAAAATAACAAGTGGTTGGAGAGATGGCGCTGTACTTAAGAGACTTGCTGCTCTTCCAGAGGGCC
1331 TGAGTCCAGTTCCCAGCACCCACACTGAGTGGCTCACAACTACCTGCAACTTCAGCTCACATGGATCTAG
1401 CTTCTGGCCCCTTGGGCATTTGTGGACGAGTCCAAACACAAATAAAGATTAAAAAACAAAACAAAACAAA
1471 AACAAAAACCAACCCTTTTGTAAAAAGCAAACAAAAAGAACCATCATCTAATGAAGGTGACATGGACACT
1541 GCAATCTGTATTTTTACTTTTGTTGTTATATTTTTATTTTATTATTTTTTTTTTTTGGGTGGGTGGGTGGG
1611 GTGTTGANACAGGGTTTCTCTGTGTAGCCCTGGCTGTCCAGGAACTCACTCTATAGGCCAGGCTGGCCTC
1681 GAACGCAGAAATCCTCTGGCCTCTGCCCATCATATTTTTATTCTTTAAAAAAAAAAAAAAAAAGGGCAGA
1751 GGTCGGAGAGATGTCTCAGTGGTTAAGAGCACAGACTGCTCTTCTGAAGGTCCTGAGTTCAAATCCCAGC
1821 AGCCAGGTGAGTTCAAAACTCACAACCATCTGTAATGAGATCCGATGTCCTCTTCTGGAGTGTCTGAAGA
1891 CAGCTGCAGTGTACTCACATATGATAAATAAATAAATCTTTTAAAACAAGTCACATGCCGGGCATGGTGG
1961 CGCACACCTTTAATCCCAGCACTTGAGAGGCAGAGGCAGGCGAATTTCTGAGTTCAAGGGCAGCCTGGTC
2031 TACAGAGTGAGTTCTAGGACAGCCAGAGCTATACAGAGAAACCCTGTCTCGAAAAACCAAAAAAAAAAAA
2101 GAAAGAAAAAAAAGAAAAAAAAAGTCACAGGCCAGAGAGATTGCCGAGCAGAGCACCCACTGGTCTTCTAG
2171 AACAATGGTGCTCAACCAGTGGGTCTCCAATGGCTTTGACAGGAGTCACCTAAGGACATCCGAAAACACA
2241 GTAGTTATATTAGAATTCATAGCAGTAGCAAATTACAGTTAGGAAGTAGTGGGGTCACTGCACCATGAAG
2311 AATTGTATTAAAGGGCAGCAGCGTTAGGAAGTTGAGAAGCACTGGACTGGAGGCTATGAATCCAGTTCGC
2381 ACACCCATGCTAGACAGCTTACAGTGGTTAGGCTCCAGTGGACTGGACACTCCCCAACCCCACTGTTGCC
2451 ACACCCTAGTCCTTTTCTCCTCCTCCTTTCTTTTATTTTCTTCTGGTTTTTCAAGACAGGGTTTCTCTAT
2521 AGCCCTGGTGTTCTCAAACTCAGAGGTCCACCTGCCTTAGCCTCCCCAGTGCTAGGACTATAGGTGTGTG
2591 GCACCAAGCATATTTATTTGTATGTATGTAGGTATGCAGAGGACAACTGGGAGTCAGCTTGTTTTCCAAC
2661 TTGTTGAGGTGGGTGGGGTCTCTTACTGTTGCTACACTGTGTAATCCAGGCTAGTTTGTTGACAAGCCTC
2731 TGTAGTACATCTCCCTATAGCAGTGCTCGATTACAGATTGTACCACCAAATTTAGCTTTTGCCTCGGTTT
2801 GGGGGCTTGAACTCAGGTTGTCAGGTTTGTATGGCTAGTGTTTTAACAGGCCAAACCATCTCTGCAACCC
2871 TGAAACTGGTACGTTAAACAAACTCTCACCATTCCAACTAACATGTAAGAACTAGGTACGTGAGCCAGGA
2941 TATTAAAGGGGAAAGTACTTGCCTGACAGTGTGCGGCACTGGGTTCTACTCTCAGAAACACAAACAAAAG
3011 AAAGAAACAAAGAGAAAGAGAGAGAACACATTTTTTAGATCTTCAAGTACTTCTAGGAGGAATCATTTTT
3081 ACTACAACTCTATATAAAAATCCTCACAAGGGCCGGGCGGTGGTGGCGCACGCCTTTAGTCCCAGCACTT
3151 GGGAGGGCAGAGGCAGGCGGATTTCTGAGTTCAAGGCCAGCCTGGTCTACAAAGTGAGTTCCAGGGCAGC
3221 CAGGACTACACAGAGAAACCCTGTCTCAAAAACCAGAAAAAAAAAAAAACAAACAAAACAAAACAAAAAA
3291 AAAAACCTCACAAGGCAGAAGAGTCCACAGCTAATTAAGATGCCCAAAGTCAAAGCTCAGCTCTTCCAAC
3361 CCTAGAGCTGGAGCAGGACTCTATACAATCACTCTTTGGCTCACTTAAAATTCTTCAAGGAAAATCAGGA
3431 CACTTCCCAGGACAAAGATCAGTACTTCTCTCTAACCCATCTCTCTCCTGCTCAGCTCTGTCCCATCCCT
3501 CACTTCCACTGCAAGGCTCCACACCTCCAGGCCTCCTGTCTTCGTTCTAGACTTCAGTTCCTTCCATGCT
3571 AGTCATTTATACTANCAGTTCTACAGTGGAAACTGCTACCCCTCAGTCTCCTGACCTCTGTATCTCTGTA
3641 TTTAACTCGTTGGTTACTTGGGTCTTGCTCCCACACACTTCCTCTATAAGGCTGTCTTTCTTTTTTTTCT
3711 TTAAAGACTGTATTTCCTCTCTTTGTTTATGTGTATGCACACTCACTAGAGGGTGTCGAGGGCAGAGGAC
3781 AGCTTGCACAGTCAGTTCTCTCCTGTTACTAGATGGCTCTAGGAGGCTAAACTCAGGTCACCAAGCTT
```

Shaded regions represent repeat sequences. Poly(A) tracts are underlined.

Appendix 5 Nucleotide sequence of pλ-27BD (~2.6kb)

AAGCTTTTGG GGCTGAAAAG ATGGCTCAAG CTACATCCCT AATACTTATG TAAAAGTCAG
GAGGCATGNT AGTGCTTATA TGGAACACCA GCTTGGGAAG GTGGGGATCC AAACAGATGA
ATCTCCAGCT CACTCCTCAC TGGCTGGCTC CTCAGTGTAG TGAATGAGAA AGATCCAGGC
TCAGAGAGAG CAGCCACTGT CAGAGAGAGA GAGAATGAGG GAGGGAGGGA GGGAGGGAGG
GAGGGAGGGA GGGAGGGAGG GAGGGGGGAG GGAGAGAGAA AGAGAGAGAG AGAGAGATCC
AGTGTGTACT TGTGCTCGCA TGAGTGTGCG CATACACACA CACACACACA CACACACACA
CACACACACA CACACACGGT TTTTCACTGC TCCAGTGTGC AGTCTCCATA GCAAAGTTTT
CCAAGTTCCA GATTGCCATT TGTGCAGCAG GTGTGAGGGG CCACAGCTGG GTGAGCGTCT
CCTTGACCTG CTTGTTCATG CAGGGCTTGG GACCTGTGGG TTTACTTACA TAGAGACTGA
CCACAGTGTC AGTCCGATGG TGACCAAGTC CTTGAAGCTA GCATGCAGTG TGAACAGGGC
TATGCACAAT GAACCTGACT GCAAATGCAA ATCCTAATAC TTAACTAGAC CTGAAACCTC
AGCACTTGAG AAGCACAGGG AGGGCTGGAG AGATGGCTCA GAGGTTAGGA GCACTGACTG
CTCTTCCACA GGTCCTGAGT TCAATTCCCA GCAACCACAT GGTGGCTCAC AACCATCTGT
AATGGGGATC TGATGCCTTC TTCTGGTGTG TCTGAAGACA GCGACAGTGT ACTCATATAA
ATAAAATAAA TCTTTTTTTA AAAAAGGAGA GACAGCTGGG AAGACGGCTC AGTGGTTAAG
AGCGCCGACT GCTCTTCCGA AGTCCCGAGT TCAAATCCCA GCAACCACAT GGTGGCTCAC
AACCATCCGT AACAAAATCT GATGCCCTCT TCTGGAGTGT CTGATAACAG CTACAGTGTA
CTTACATATA ATAAATAAAT AAATAAATAA ATAAAATTAA AATTAATTAA TTCATTCACA
CCGGAGAGAG AGAGGCACAG GGaGGCGCAT TGGGGGCTAG CCTCAGCTAC ACGAAGAGTT
GGAAGCCAAC CTAGACTACA TAAGAGTGTC TCGAAGAAGA AGAAGAAGAA GA

CATCCACCTC TCTGTCAGCA GGAAGCCCAG AGAAGGAGCT GAGAGGGGGC CTGAGGTATT
GGGTTGGCAG AAACAGGTTA CTGAGAGGAT CTGGTGGGGG AGATGAGGAA GCCAGGTGGG
GAAAGAGAGA AAGTCACCAC AGATGTGAGA AAGAGAGGCA TCCTGGAGAG CAACAGGGTA
AGAGCTGAGG GGACTTTGTG AGGTAGGTGG CACTTGGCCT GAAGGACCTC ATAGGGCTTG
TGGGATGGCT CAGCAATTGA GAGCACTTGT CCTTCCAGAA AACCCAGGTT CACTTGGTGG
CACTACCTGG TAACTCCAGT GTCTGATGCT TTCTTCACAT CTGTAAGCAC CAAGCACTCA
CCTGGTGCAC ATACCTGTGG AATATGCACA CTCACAAACA GACGGAGAGA TAGATAGATA
GATAGGTAGG TAGGTAGGTA GATAGATAGG TAGGTAGGTA GGTAGGTAGA TAGATAGATA
GATAGATAGA TAGATAGATA GATAGATAGA TAGATAAGAG TCAGGCAGTG GCAGCTCACA
CTAATGCCAG CATTAGGGAG GCAGAAGCAG GTGGATCTCT GAGTTCAAGG CTAGCCTGGT
TTACAGAGGG AGTTCCAGGA CACCCAGGTA TATACAGAGA AACCCTGGAG AGAGAGAGAG
AGAGAGAGAG AGAGAAGGAG AAGAAGGAGG AGGAGGAGGA GGAGAAGGAG AAGAAGAAGA
AGAAGAAGAA GAAGAAGAAG AAGAAGAAGA AGAAGAAGAA GAAGAAGAAG AAGAGAGAGA
AAAAAGAAAA GAATGGGCCC CATATCGTGT TGTGCTTGGC AAAGTCCATG CTTATCTGAG
AATATCCTCA GGTAGAGAAT TTCAGGTACA CATGAGTCCC CAGACCTTTT GCTGACAACC
ATGTGTGTGT ATTTTATTGT CTTTATAACA AAATCAGTTC AGAACTGTAT CTCTCTCTCT
CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCACACA CACACACACA CACACACACA
CACACACACA CTCCTCCAAG CT

Shaded region represents repeat sequences. The $(CT)_n(CA)_n$ repeats have been boxed and imperfect tandem repeats $(GAAG)_n(GA)_n$ and $(TAGA)_n(TAGG)_n$ are heavily underlined. The imperfect poly(A) tract $[(TA_n)]_y$ in the repeat sequence is lightly underlined. The gap between the two blocks of sequences represent ~400bp of unsequenced DNA.

Appendix 6 The nucleotide sequence of pλ-7G (2.815kb)

```
   1 CAATCATAAT GAAGTGGCAC GACAGGTTCC CGACTGGAAA GCGGGCAAGT GACCGAACGC
  61 AAATTAATGT GAGTAGCTCA CTCATTAGGC ACCCCAGGCT TTACACTTTA TGCTTCCGGC
 121 TCGTATGTTG TGTGGAATTG TGAGCGGATA ACAATTTCAC ACAGGAAACA GCTATGACCA
 181 TGATTACGCC AAGCTATTTA GGTGACACTA TAGAATACTC AAGCTATGCA TCCAACGCGT
 241 TGGGAGCTCT CCGGATCCAA GCTTCTTCCT CCTCCTCCTC CTCTTCCTCC TCCTCCTCCT
 301 CTTGTCTCCT CTTCTGTCTC CTCTTCCTTC TCCTCCTCTT CCTCCTCTTG CTCCTCCTTT
 361 CCCTCCTCCT TCTTTATTCC AAACAGGTTG GGNCTATGCT GTTCAGGCTG GCTTAGGACT
 421 CACTATGTAG CCCACGCTAG ACTTGAATCT GCTATGAACC TTCTGCCTCA GCTTCTCCTG
 481 CCCACTCCCT ACTGAAAGAC CCCTTCATAA GGCTTAGCCA GCTACCTGCA GTAACGCCAT
 541 TTTGCAAGGC ATGGGAAAAT ACCAGAGCTG ATGTCCTCAG AAAAACAAGA ACAAGGAAGT
 601 ACAGAGAGGC TGGAAAGTAC CGGGACTAGG GCCAAACAGG ATATCTGTGG TCAAGCACTA
 661 GGGCCCCGGC CCAGGGCCAA GAACAGATGG TCCCCAGAAA TAGCTAAAAC AACAACAGTT
 721 TCAAGAGACC CAGAAACTGT CTCAAGGTTC CCCAGATGAC CGGGGATCAA CCCCAAGCCT
 781 CATTTAAACT AACCAATCAG CTCGCTTCTC GCTTCTGTAC CCGCGCTTAT TGCTGCCCAG
 841 CTCTATAAAA AGGGTAAGAA CCCCACACTC GGCGCGCCAG TCCTCCGATA GACTGAGTCG
 901 CCCGGGTACC CGTGTATCCA ATAAAGCCTT TTGCTGTTGC ATCCGAATCG TGGTCTCGCT
 961 GAACCTTGGG AGGGTCTCCT TCAGAGTGAT TGACTGCCCC AACCTGGGGT CTTTCATTTG
1021 GGGCTCGTCC GGAATTTGAA AACCCCCGCC CAGGGACCAC CGACCCACCG TCGGGAGGTA
1081 AGCTGGCCAG CGATCGTTTT GTCTCCGTCT CTGTCTTTGT GCGTGTGTGT GTGTGTGCCG
1141 GCATCTACTT TTTGCGCCTG CGTCTGATTC TGTACTAGTT AGCTAACTAG ATCTGTATCT
1201 GGCGNCTCCG TGGAAGAACT GACGAGTCCG TATTCCCGAC CGCAGCCCTG GGAGACGTCT
1261 CAAGAGGCAT CGGGGGCCCC GCTGGGTGGC CCAATCAAGT AAAGTCCGAG TCCTGACCGA
1321 TTCGGACTAT TTGGGGCCCC TCCTTTGTCG GAGGGGTACG TGGTTCTTTT AGGAGACGAG
1381 AGGTCCAAGC CCTCGCCGCC TCCATCTGAA TTTTTGCTTT CGGTTTTTCG CCGAAACCGC
1441 GCCGCGCGTC TTGTCTGTCT CAGTATTGTT TTGTCATTTG TCTGTTCGTT ATTGTTTTGG
1501 ACCGCTTCTA AAAACATGGG ACATACCGTT ACCACCCCTC TGAGTCTGAC CCTAGAACAC
1561 TGGGAAGATG TCCAGCGCAT CGCGTCCAAT CAGTCCGTAG ATGTCAAGAA GAGACGCTGG
1621 GTCACCTTCT GCTCTGCCGA GTGGCCAACT TTCGGTGTAG GGTGGCCACA AGATGGTACT
1681 TTTAATTTGG ACATTATTCT ACAGGTTAAA TCTAAGGTGT TCTCTCCTGG TCCCCACGGA
1741 CACCCGGATC AGGTCCCATA TATTGTCACC TGGGAGGCTA TTGCCTATGA ACCCCCTCCG
1801 TGGGTCAAAC CTTTTGTCTC TCCCAAACTC TCCCCCTCTC CAACCGCTCC CATCCTCCCA
1861 TCCGGTCCTT CGACCCAACC TCCGCCCCGA TCTGCCCTTT ACCCTGCTCT TACCCCCTCT
1921 ATAAAACCCA GACCTTCTAA ACCTCAGGTT CTCTCCGATA ATGGCGGACC TCTCATTGAC
1981 CTTCTCTCAG AAGACCCTCC GCCGTACGGA GGACAGGGAC TGTCCTCCTC TGACGGAGAT
2041 GGCGACAGAG AAGAGGCCAC CTCCACTTCT GAGATTGCTG CCCCCTCTCC CATAGTGTCT
2101 CGCCTGCGGG GCAAAAGAGA CCCCCCCGCG GCAGATTCCA CCACCTCTCG GGCTTTCACA
2161 CTCCGTTTGG GGGGTAATGG TGAGTTGCAG TACTGGCCGT TTTCCTCCTC TGATCTATAT
2221 AACTGGAAAA ATAATAATCC TTCCTTCTCT GAGGAACCAC AGGTAAACTG ACTGCATTGA
2281 TTGAATCCGT CCTCACCACC CACCAGCCCA CCTGGGATGA TTGCCAGCAA TTATTAGGGA
2341 CTCTGCTTAC CGGGGAGGAG AAGCAGCGGT TGCTCCTGGA AGCCCGAAAG GCTGTCCGGG
2401 GCAACGATGG GCGCCCCACC CAACTGCCCA ACGAGGTTGA CGCTGCTTTT CCCCTTGAAC
2461 GTCCCGATTG GGATTACACC ACCCAAAGAG GTAGGAACCA CCTAGTTCTC TATCGCCAGT
2521 TGCTTTTAGC AGGTCTCCAA AATGCGGGCC GAAGCCCCAC CAATTTGGCC AAGGTAAAAG
2581 GAATAACCCA GGGACCTAAT GAGTCCCCCT CAGCCTTCCT AGAGAGACTC AAGGGGAAAC
2641 CTATCGCAGA TACACTCCTT ATGATCCTGA GGACCCTGGG CAAGAAACGA ATGTATCTAT
2701 GTCATTCATC TGGCAGTCCG CTCCAGACAT TGGTCGAAAG TTAGAGCGGT TAGAAGACTT
2761 AAAAAGTAAA ACTTTAGGAG ATTTAGTGAG AGAAGCCGAA AGGATCTTTG AGAT
```

Shaded region represents genomic DNA sequence and boxed region represents the LTR. Partial sequence of the viral *gag* gene follows.

Appendix 7 Nucleotide sequence of flanking genomic DNA in pλ-7G and the corresponding amino acid sequences (single letter code) translated in six frames.

a) 5' to 3' (plus strand)

```
          10        20        30        40        50        60        70
5'         *         *         *         *         *         *         *
  AAGCTTTTCCTCCTCCTCCTCTTCCTCCTCCTCCTCCTCTTGTCTCCTCTTCTGTCTCCTCTTCCTT
      F  S  S  S  S  S  S  S  S  S  S  S  S  C  L  L  F  C  L  L  F  L>
   K  L  F  L  L  L  L  L  F  L  L  L  L  L  S  P  L  L  S  P  L  P>
     A  F  P  P  P  P  P  L  P  P  P  P  P  L  V  S  S  S  V  S  S  S  F>

          80        90       100       110       120       130       140
           *         *         *         *         *         *         *
  CTCCTCCTCTTCCTCCTCTTGCTCCTCCTTTCCCTCCTCCTTCTTTATTCCAAACAGGTTGGGNCTATGC
   L  L  L  F  L  L  L  L  L  L  S  L  L  L  L  Y  S  K  Q  V  G  X  M>
  S  P  P  L  P  P  L  A  P  P  F  P  P  P  S  L  F  Q  T  G  W  X  Y  A>
    S  S  S  S  S  S  C  S  S  F  P  S  S  F  F  I  P  N  R  L  G  L  C>

         150       160       170       180       190       200       210
           *         *         *         *         *         *         *
  TGTTCAGGCTGGCTTAGGACTCACTATGTAGCCCACGCTAGACTTGAATCTGCTATGAACCTTCTGCCTC
  L  F  R  L  A  *  D  S  L  C  S  P  R  *  T  *  I  C  Y  E  P  S  A  S>
    V  Q  A  G  L  G  L  T  M  *  P  T  L  D  L  N  L  L  *  T  F  C  L>
   C  S  G  W  L  R  T  H  Y  V  A  H  A  R  L  E  S  A  M  N  L  L  P>

         220       230       240       250
           *         *         *         *
  AGCTTCTCCTGCCCACTCCCTACTGAAAGACCCCTTCATAAGG  3'
    A  S  P  A  H  S  L  L  K  D  P  F  I  R>
   S  F  S  C  P  L  P  T  E  R  P  L  H  K  E>
  Q  L  L  L  P  T  P  Y  *  K  T  P  S  *  G>
```

b) 5' to 3' (minus strand)

```
          10        20        30        40        50        60        70
5'         *         *         *         *         *         *         *
  CCTTATGAAGGGGTCTTTCAGTAGGGAGTGGGCAGGAGAAGCTGAGGCAGAAGGTTCATAGCAGATTCAA
     L  W  R  G  L  S  V  G  S  G  Q  E  K  L  R  Q  K  V  H  S  R  F  K>
    L  M  K  G  S  F  S  R  E  W  A  G  E  A  E  A  E  G  S  *  Q  I  Q>
   P  Y  E  G  V  F  Q  *  G  V  G  R  R  S  W  G  R  R  F  I  A  D  S>

          80        90       100       110       120       130       140
           *         *         *         *         *         *         *
  GTCTAGCGTGGGCTACATAGTGAGTCCTAAGCCAGCCTGAACAGCATAGNCCCAACCTGTTTGGAATAAA
    S  S  V  G  Y  I  V  S  P  K  P  A  W  T  A  *  X  Q  P  V  W  N  K>
   V  *  R  G  L  H  S  E  S  *  A  S  L  N  S  I  X  P  T  C  L  E  *>
  S  L  A  W  A  T  *  W  V  L  S  Q  P  E  Q  H  X  P  N  L  F  G  I  K>

         150       160       170       180       190       200       210
           *         *         *         *         *         *         *
  GAAGGAGGAGGGAAAGGAGGAGCAAGAGGAGGAAGAGGAGGAGAAGGAAGAGGAGACAGAAGAGGAGACA
   E  G  G  G  K  G  G  A  R  G  G  R  G  G  E  G  R  G  D  R  R  G  D>
  R  R  R  R  E  R  R  S  K  R  R  K  R  R  R  R  K  R  R  Q  K  R  R  Q>
    K  E  E  G  K  E  E  Q  E  E  E  E  E  E  K  E  E  E  T  E  E  E  T>

         220       230       240       250
           *         *         *         *
  AGAGGAGGAGGAGGAGGAAGAGGAGGAGGAGGAGGAAAAGCTT  3'
  K  R  R  R  R  R  K  R  R  R  R  R  K  S  F>
    E  E  E  E  E  E  E  E  E  E  E  E  K  L>
   R  G  G  G  G  G  R  G  G  G  G  G  K  A  X>
```

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