Molecular Biological Analyses of Feline Immunodeficiency Virus

Keizo TOMONAGA

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PREFACE

History of feline immunodeficiency virus and significance of this study

In 1986, a feline T-lymphotropic virus was isolated from a cat with an acquired immunodeficiency syndrome (AIDS)-like disease in the United States (Pedersen *et al.*, 1987). This virus appeared to be morphologically similar to human immunodeficiency virus (HIV) by electron microscopy, showed Mg^{2+}-dependent reverse transcriptase activity, and induced cytopathic effects in feline T lymphocytes. From these properties, this virus was classified to genus lentivirus of the retrovirus family and designated as feline immunodeficiency virus (FIV). Subsequently, isolation of the virus was reported in various countries including Japan (Haubour *et al.*, 1988; Miyazawa *et al.*, 1989*a*; Morikawa *et al.*, 1991*b*; Rigby *et al.*, 1993; Siebelink *et al.*, 1992). Several seroepidemiological surveys were also revealed that FIV has spread worldwide and the incidence of the FIV infection in cats varied from between 1% and 15% in healthy cats to between 3% and 4% in diseased cats in various countries (Bennett *et al.*, 1989; Cohen *et al.*, 1990; Furuya *et al.*, 1990; Gridem *et al.*, 1989; Gruffydd-Jones *et al.*, 1988; Hosie *et al.*, 1989; Ishida *et al.*, 1988; Lutz *et al.*, 1990; Sabine *et al.*, 1988; Shelton *et al.*, 1989, 1990; Swinney *et al.*, 1989; Yamamoto *et al.*, 1988).

Shortly after the virus isolation, molecular cloning and sequencing of the FIV genome were reported by Olmsted *et al.* (1989*a*, *b*), and Talbott *et al.* (1989), who suggested

that genomic organization of the FIV appears to be relatively simple compared with that of primate lentiviruses, in that the gene contains at least three short open reading frames (ORFs), putative *vif* (or ORF-1), ORF-A (or ORF-2), and ORF-B (or ORF-4), in addition to the structural genes common to all retroviruses. In Japan, Miyazawa *et al.* (1991) also reported a molecular cloning of FIV which was biologically and genetically different from the original U.S. isolate. These observations indicated that the genomic heterogeneity was also present among FIV strains, as like in the case of HIV or SIV isolates (Alizon *et al.*, 1986; Benn *et al.*, 1985; Kestler *et al.*, 1988; Li *et al.*, 1989; Zagury *et al.*, 1988)

At the time that research described in this thesis started, only a little was known about molecular biology of the FIV except for the reports described above. There was no functional information on the putative *vif*, ORF-A and other genes including structural genes. To elucidate the role of these genes was important subjects for understanding the life cycle and pathogenicity of the virus, the molecular and biological analyses of the FIV described in this thesis were carried out. In CHAPTER 1, the author described the biological and biochemical analyses of the putative *vif* gene which involved in the infectivity of the virus. In CHAPTER 2, mutational analysis was carried out to understand the

role of the ORF-A gene, and the author demonstrated that the ORF-A gene plays an important role in the viral replication. In this chapter, moreover, analysis of the functions of the other genes were described.

The lentiviruses encode *trans*-acting regulatory genes, *tat* and *rev*, which are essential for efficient virus replication (Pavlakis and Felber, 1990). In 1991, our group reported that a short 3' ORF that overlaps the long terminal repeat (LTR), ORF-B, has a *rev* gene activity and at least a part of *rev* gene (Kiyomasu *et al.*, 1991). However, the detail splicing pattern of the *rev* gene was unclear. Furthermore, identifying and cloning of cDNAs of the FIV were necessary for more detail analysis of the FIV genes including the *rev* gene. Thus, the author performed the analyses of the expression and splicing pattern of the FIV gene. In this experiment, the author cloned the several cDNAs which were useful for the researches of the FIV genes. The results were described in CHAPTER 3. In CHAPTER 4, the author analyzed roles of the *rev* gene which involved in species- or cell-specific tropisms of FIV by using cDNAs which obtained in CHAPTER 3.

In the section of REVIEW, the author overviewed regulation of expression of FIV including the data of this thesis as well as the recent information from our and other groups.

In the section of CONCLUSION, the author summarized and discussed the results presented in the four chapters. Some most recent, unpublished data supplying additional information, are included.

REVIEW

Regulation of expression of feline immunodeficiency virus

A: INTRODUCTION

Feline immunodeficiency virus (FIV) belongs to the genus lentivirus, *retroviridae* and is considered to be an useful animal model for acquired immunodeficiency syndrome (AIDS) of human that caused by human immunodeficiency virus (HIV). FIV has all general, structural and functional characteristics of this class of the virus (Cullen, 1991, 1992). The FIV genome (Fig. 1) contains the three structural characteristic retroviral genes, *gag*, *pol*, and *env*, which encode the structural viral components. The mRNAs of structural genes are expressed with unspliced or singly spliced forms to cytoplasm (CHAPTER 3 in this thesis). These three genes produce precursor polyproteins that are subsequently assembled and processed to produce virions, which exit the cell by budding. The virion is surrounded by a glycolipid "envelope" originating from the cellular plasma membranes. The viral Env glycoproteins are embedded in this membrane and protrude to the outside of the virion. The core in viral particle contains the $p24^{gag}$ capsid protein, the viral enzymes, reverse transcriptase (RT), integrase, protease, and dUTPase (Elder *et al.*, 1992, 1993), and two copies of viral RNA bound to the nucleocapsid protein.

In addition to the structural genes, three other viral genes (*vif*, ORF-A, and *rev*) are identified in the genome

(Fig. 1). *vif* is important for the cell-free virus infectivity (CHAPTER 1 in this thesis). One of these genes, *rev* is essential regulatory factor because viral replication cannot proceed in its absence (Kiyomasu *et al.*, 1991; Phillips *et al.*, 1992). Although the ORF-A gene is similar in location and sequence to the *tat* gene of the other lentiviruses (Maki *et al.*, 1992; Olmsted *et al.*, 1989*a*, *b*; Talbott *et al.*, 1989), the gene is not essential for viral replication in established T-cell lines and feline primary peripheral blood lymphocytes (PBLs) (CHAPTER 2 in this thesis).

The purpose of this review is to summarize the molecular events in the expression of FIV comparing with other lentiviruses, especially with HIV-1 and visna virus serving as respective prototypes.

B: LIFE CYCLE OF FIV

The life cycle of FIV is depicted in Fig. 2. Initially, infectious virus particles are considered to bind to the specific FIV receptor via the surface glycoprotein. Until now, however, there is no report that the specific factor(s) on the cell surface binds to the FIV surface glycoprotein. The binding between the virus surface glycoprotein and cellular specific factor(s) results in the fusion of viral

and cellular membranes following by the penetration and entrance of the viral core into the cells. This is apparently the same mechanism that leads to giant syncytia formation, which commonly observed in the FIV infection (Miyazawa *et al.*, 1989*b*, 1990). The enzyme RT converts the viral RNA to DNA (Baltimore, 1970; Cronn *et al.*, 1992; North *et al.*, 1990*b*; Temin and Mizutani, 1970), which enters the nucleus and integrates into cellular DNA. This RT activation step is considered to be one of the drug therapy targets of the lentivirus infection including FIV (Hayes *et al.*, 1993; North *et al.*, 1989, 1990*a*; Remington *et al.*, 1991). Integration initiates the late phase of viral life cycle. The proviral DNA integrated into the cellular genome behaves as cellular genes and uses the cellular machinery for the production of new virus. Like all cellular genes encoding proteins, the virus is transcribed into mRNA by the cellular RNA polymerase II . Both viral and cellular factors are essential for viral replication and regulate viral expression at transcriptional and post-transcriptional levels. The structural proteins assemble into particles containing the viral enzymes and two copies of the unspliced mRNA, which also serves as genomic RNA. The particles bud from the cell surface and mature through the action of viral protease.

FIV can accomplish the life cycle in T lymphocytes, monocytes/macrophages, follicular dendritic cells and brain cells (Brown *et al.*, 1991; Brunner and Pedersen, 1989; Dow *et al.*, 1991; Kawaguchi *et al.*, 1992*a*; Miyazawa *et al.*, 1989*a*, *b*; Toyosaki *et al.*, 1993; Yamamoto *et al.*, 1988). However, there are several limiting steps precluding the normal viral life cycle described above. The first limiting step exists in the viral entry, which is involved the presence of a number of specific factors. In the infection of the HIV-1 to the target cells, the CD4 molecule play an important role as a HIV-1 receptor (Dalgleish *et al.*, 1984; Landau *et al.*, 1988). It has been reported that some strains of FIV have tropism for the CD4 positive T-lymphocytes in vitro (Miyazawa *et al.*, 1989*a*; Phillips *et al.*, 1990; Siebelink *et al.*, 1992; Tokunaga *et al.*, 1992), and feline CD4 positive cell line lost their reactivity to monoclonal antibodies against CD4 molecule after persistent infection with FIV (Tokunaga *et al.*, 1992). These data suggested that feline CD4 molecule is also important factor of the FIV infection. However, it was also reported that feline CD8 positive T-cells is also one of the target cells of the FIV infection in vitro (Brown *et al.*, 1991), and a highly lymphotropic FIV isolate (strain TM1) could not accomplish the early phase of the virus life cycle (entry to integration) in a feline non-lymphoid cell, Crandell feline

kidney (CRFK) cells, on which feline CD4 molecules were expressed (Norimine *et al.*, 1993). Because the entry manner of FIV to target cells remains unclear yet, identifying it is important for full understanding of the virus life cycle.

Another limiting step of viral replication in the cells exists in the viral gene expression process. It is anticipated that the complex regulation of virus expression will be explained through the understanding of the interaction of viral components and regulatory factors with the cellular machinery. Transcriptional activity of the FIV promoter is important for the levels of virus production and replication (Miyazawa *et al.*, 1992*a*). In addition, post-transcriptional mechanisms involving viral regulatory genes are important for the intricate interactions of FIV with its host. Thus, regulation of the viral expression strategy will be described below.

C: VIRAL EXPRESSION STRATEGY

The production of many proteins including structural proteins from the unique FIV promoter is achieved by three different mechanisms; ribosomal frameshifting (for the production of Gag-Pol polyprotein) (Morikawa *et al.*, 1992), alternative splicing, and production of bicistronic mRNA. All three mechanisms are used in other lentiviruses

including HIV and visna virus (Clements and Wong-Staal, 1992*b*; Pavlakis and Felber, 1990).

Ribosomal frameshifting is one of the mechanisms that regulates the expression of some proteins during the translation of two overlapping genes from a single mRNA. The *pol* genes of a majority of retroviruses are translated as *gag-pol* fusion proteins by ribosomal frameshifting within the *gag-pol* overlapping region (Hizi *et al.*, 1987; Jacks and Varmus *et al.*, 1985; Jacks *et al.*, 1987, 1988*a*, *b*; Moore *et al.*, 1987; Wilson *et al.*, 1988). FIV contains a consensus frameshift signal sequence of GGGAAAC within the *gag-pol* overlap region followed by a potential pseudoknot tertiary structure. Morikawa *et al.* (1992) suggested that the ribosomal frameshift in the FIV *gag-pol* region required the precisely positioned downstream pseudoknot structure and that the level of scanning speed of the ribosomes may be involved in efficient frameshifting.

The transcriptional and translational manners of viral mRNAs were well studied in HIV and visna virus systems (Clements *et al.*, 1990; Davis *et al.*, 1987; Schwartz *et al.*, 1990*a*; Viglianti *et al.*, 1990). The author has detected that infection of feline lymphoblastoid cell lines with FIV results in the production of more than 10 different species of the viral mRNAs, and detail conformations of the transcription pattern of FIV mRNAs are described in CHAPTER

3 in this thesis. The primary full-length viral RNA transcript is processed into various mRNAs by multiple splicing events and by a mechanism that appears to involve temporal regulation of gene expression (Cullen and Greene, 1990). In the early phase of the virus infection, viral encoding regulatory factors, such as Tat and Rev, are expressed. These viral products *trans*-activate virus gene expression or alternative splicing of viral mRNAs via *cis*-acting RNA target sequences within the viral genome. To date, although the Tat *trans*-activity is not detected in the FIV genome, it was demonstrated that the FIV Rev also *trans*-activates the expression of late phase genes including the three structural genes (Kiyomasu *et al.*, 1991; Phillips *et al.*, 1992; CHAPTER 3 in this thesis).

Although mRNAs producing more than one protein (bicistronic) are not common in eukaryotic systems, several HIV mRNAs are bicistronic, including all the mRNAs producing Env (Schwartz *et al.*,1990*a*, *b*, 1992). Because of alternative splicing and bicistronic mRNAs, most HIV proteins are produced by more than one mRNAs. In the cases of visna virus, equine infectious anemia virus (EIAV), and bovine immunodeficiency virus (BIV), some bicistronic mRNAs were also detected (Davis *et al.*, 1987; Noiman *et al.*, 1990; Oberste *et al.*, 1991). In the FIV, the transcript which has a bicistronic coding capacity was detected both in the

Japanese (TM2) and in the American (Petaluma) isolates (Phillips *et al.*, 1992; CHAPTER 3 in this thesis). These bicistronic mRNA may be a characteristics of lentiviral translation manners. Additionally, some of the FIV mRNAs, like as HIV and visna virus mRNAs, are spliced two or three times (Phillips *et al.*, 1992; CHAPTER 3 in this thesis). This property is not seen in other retrovirus except the human T-cell leukemia virus (HTLV) and bovine leukemia virus (BLV) (Derse, 1987; Pavlakis *et al.*, 1988; Yoshida and Seiki, 1987), which produce one doubly spliced mRNA. This manner may also be a property of gene expression of the lentiviruses which show complex regulations.

The viral promoter

The FIV long terminal repeat (LTR) contains the several enhancer/promoter in the U3 region and the polyadenylation signal in the R region, which are essential for the viral transcription in the host cells (Fig. 3).

The HIV promoter interacts with several cellular transcription factors such as NF-κ B and Sp1 (Fig. 3) (Franza *et al.*, 1988; Jones *et al.*, 1988; Jones, 1989). At upstream of TATA box, there are three binding sites for Sp1 and two sites for NF-κ B. At downstream of the TATA box, there is the TAR region which is necessary for Tat protein

function (Arya, 1988; Arya and Gallo, 1986, 1988; Hauber and Cullen, 1988; Muesing *et al.*, 1987; Rosen *et al.*, 1985; Sherman *et al.*, 1988). Several other proteins that interact with the HIV LTR have been identified (TCF-1, HIVEN86A, EBP-1, HIP, LBP-1, CTF/NF-I) (Jones, 1989). Visna virus and caprine arthritis encephalitis virus (CAEV) have four AP-1 and one AP-4 binding sites (Fig. 3). Interaction between NF-κ B and the HIV LTR and that between cellular factors and the AP-1 binding sites in the visna virus LTR have been shown to be important for transcriptional activity in T-cell and macrophage cell lines, respectively (Gabuzda *et al.*, 1989; Hess *et al.*, 1989; Wu *et al.*, 1988; Zeichner *et al.*, 1991). The EIAV LTR contains the binding sites for the octamer binding factor (Carpenter *et al.*, 1991; Derse *et al.*, 1988; Sherman *et al.*, 1989) and TAR region in the R region (Carvalko and Derse, 1991).

Transcriptional activation of FIV is also controlled by core enhancer elements in the U3 region, that contain the binding sites for the one AP-1 and two AP-4. Recently, it was demonstrated that this region was important for basal promoter activity in feline non-lymphoid cell lines, such as CRFK cells and G355-5 cells (Kawaguchi *et al.*, 1992*b*; Miyazawa *et al.*, 1993; Sparger *et al.*, 1992). Furthermore, Miyazawa *et al.* (1993) demonstrated that the FIV LTR could be activated by the expression of FOS which is cellular

transcriptional factor recognizing AP-1 binding site. However, the AP-1 binding site was not essential for viral replication in T-lymphoblastoid cell lines (Miyazawa *et al.*, 1993). In visna virus, it was reported that FOS and JUN activated the virus gene expression in macrophages through an AP-1 binding site (Shih *et al.*, 1992). In addition to the AP-1 and AP-4 binding sites, several putative binding sites for enhancer/promoter proteins such as NF-κ B, IRF, C/EBP, ATF, TFII D (TATA box), and LBP-1 are present (Fig. 3) (Maki *et al.*, 1992; Miyazawa *et al.*, 1991; Phillips *et al.*, 1990).

The role and significance of most of these interactions are still unknown. It is anticipated that expression of the viral promoter in various cells would be different depending on the availability of the appropriate factors which themselves are regulated by the physiological status and differentiation stage of each cell. In addition to the enhancer/promoter sequences, the FIV LTR contains a TAR-like stem-loop structure on the R region (Maki *et al.*, 1992; Morikawa *et al.*, 1991), however no evidence is available for the binding of proteins to the structure at present.

D: VIRUS ENCODING GENES

Lentiviruses have several small ORFs in their genome,

in addition to the three structural genes (*gag, pol*,and *env*) that are in common with other retrovirus. The HIV-1 has six auxiliary genes in the genome (Fig. 1). These genes are classified into "early regulatory genes" which are expressed in early phase of virus infection and "late regulatory genes" whose expression are regulated by the *rev* gene. Expression, replication, and/or infection of the virus are highly regulated by these virus auxiliary genes. On the other hand, the genomic organizations of non-primate lentiviruses including FIV are relatively simple compared with that of primate lentiviruses in that gene contains at least three auxiliary genes. (Fig. 1) (Maki *et al.*, 1992; Olmsted *et al.*, 1989*a, b*; Talbott *et al.*, 1989; Sonigo *et al.*, 1985). Although these auxiliary genes play important roles for life cycle of the viruses, its detail functions in vivo are unclear yet. In the following section, updated information of regulatory genes of the FIV are introduced.

Early regulatory genes

1) *ORF-A gene*

The ORF-A gene of FIV resembles the first coding exon of *tat* gene of primate lentivirus in its size and location on the genome (Fig. 1). The mRNAs which encode ORF-A sequence are detected in FIV-infected cell lines as doubly

or triply spliced transcript (Phillips *et al.*, 1992; CHAPTER 3 in this thesis), and express in early phase of virus infection (CHAPTER 3 in this thesis). From these observations, the ORF-A gene was predicted to be a *trans*-activator gene of the FIV, as like *tat* genes of other lentiviruses.

The Tat proteins of lentiviruses function to *trans*-activate LTR-dependent gene expression. The *tat* genes of primate lentivirus contain two exons (Fig. 1) and that mRNAs are expressed by multiple splice event. The HIV-1 Tat function is mediated through a *cis*-acting LTR sequence termed the Tat responsive element (TAR) which forms a stem-loop structure (Fig. 4). This *cis*-acting sequence is located downstream from the transcriptional start site and is thus present in all viral mRNAs (Feng and Holland, 1988; Garcia *et al.*, 1988, 1989; Hauber and Cullen, 1988; Jakobovits *et al.*, 1988; Muesing *et al.*, 1987; Selby *et al.*, 1989; Wright *et al.*, 1986). The Tat protein increases initiation rate of RNA synthesis and processibility of transcription as a result of binding with the TAR, and consequently, more viral mRNA is transcribed (Dingwall *et al.*, 1989; Feinberg *et al.*, 1986; Hauber *et al.*, 1987; Laspia *et al.*, 1989; Muesing *et al.*, 1987; Peterlin *et al.*, 1986; Rice and Matthews, 1988). In addition to this transcriptional effect of HIV-1 tat, a post-transcriptional effect has been observed (Barry *et al.*,

1991; Cullen, 1986, 1991; Feinberg *et al.*, 1986; Rosen *et al.*, 1986; Wright *et al.*, 1986).

The *tat* genes of the ungulate lentiviruses are encoded in a single exon (Fig. 1) (Davis and Clements, 1989; Saltarelli *et al.*, 1990; Stephens *et al.*, 1990). In visna virus, CAEV, and EIAV, the *tat* gene activities were also demonstrated (Dorn and Derse, 1988; Gdovin and Clements, 1992; Hess *et al.*, 1989; Saltarelli *et al.*, 1990). However, the visna virus Tat protein do not appear to function through a TAR structure and showed to *trans*-activate via a AP-1 binding site in the LTR (Davis and Clements, 1989; Gdovin and Clements, 1992; Hess *et al.*, 1989). In contrast, Tat function of EIAV is necessary TAR structure in the LTR (Carroll *et al.*, 1991; Carvalho and Derse, 1991; Dorn *et al.*, 1988, 1990; Sherman *et al.*, 1988).

In the FIV, a TAR-like structure has been identified in the R region of the LTR (Maki *et al.*, 1992; Morikawa *et al.*, 1991). In several studies, however, no *trans*-activation abilities of infectious clones were demonstrated (Miyazawa *et al.*, 1993; Sparger *et al.*, 1992). Moreover, *trans*-activity of the LTR by the ORF-A gene appeared to be quite low level in non-lymphoid cell lines (Sparger *et al.*, 1992). From these results, the functional ability of the ORF-A product as a *trans*-activator, *tat*, of the FIV LTRs was not clear. Recently, the author found that the ORF-A minus virus

reduced efficient viral replication and propagation in established T-cell lines and primary peripheral blood lymphocytes compared with wild type virus (CHAPTER 2 in this thesis). These results suggested that the ORF-A gene product plays an important role in the life cycle of the virus. Considering this observation, it is possible that the ORF-A gene product is a *trans* activator in lymphoid cells. Further studies will be needed for understanding the role of the ORF-A gene.

2) *rev*

Rev is the second necessary regulatory factor for virus expression and is encoded in all lentiviruses (Fig. 1). The Rev functions to allow the unspliced and singly spliced viral mRNAs to accumulate in the cytoplasm (Clements, 1992*a*; Steffy and Wong-Staal, 1991; Vaishnav and Wong-staal, 1991). A critical threshold of the Rev protein is believed to be responsible for the shift from the exclusive production of fully spliced mRNAs early in the replication cycle to the production of unspliced and singly spliced mRNAs needed to translate the structural proteins at late stages of virus replication (Fig. 4) (Huang *et al.*, 1991; Molim and Cullen, 1991; Olsen *et al.*, 1990). Rev protein required specific *cis*-acting RNA target sequence, termed the Rev responsive element (RRE), found in unspliced and singly spliced viral

mRNAs (Fig. 4) (Malim *et al.*, 1989*b*; Rosen *et al.*, 1988). The *rev* genes consist two exons (Fig. 1), and the second coding exon of the Rev proteins of visna virus and CAEV contains the two functional domains (RNA binding domain and activation domain), as in the case for HIV-1 Rev protein (Cochrane *et al.*, 1990; Hess *et al.*, 1986; Malim *et al.*, 1989*a*; Olsen *et al.*, 1990; Tiley *et al.*, 1990; Tiley and Cullen, 1992).

The FIV *rev* gene also consists two exons, and its mRNA is expressed doubly or triply splice event from full-length genomic RNA (Kiyomasu *et al.*, 1991; Phillips *et al.*, 1992; CHAPTER 3 in this thesis). The FIV *rev* first coding exon is overlapped and in the same reading frame as *env*, and its deduced amino acid sequence is similar to those of other lentivirus Rev proteins (CHAPTER 3 in this thesis). The first coding exons of visna virus, CAEV, and BIV *rev* also occupy similar positions at the 5' end of, and the same reading frame as, *env* (Davis *et al.*, 1987; Oberste *et al.*, 1991; Saltarelli *et al.*, 1990). The first coding exon of the putative EIAV Rev also overlaps the *env* gene but the ORF is in a different reading frame from the *env* protein (Fig. 1) (Rushlow *et al.*, 1986). The location of the RRE of FIV was located at the the 3' end of the *env* gene (Phillips *et al.*, 1992). Interestingly, the RREs of the primate lentiviruses and visna virus are located at the SU-TM junction (envelope

surface protein-transmembrane protein) within the *env* region (Lewis *et al.*, 1990; Malim *et al.*, 1989*b*; Rosen *et al.*, 1988; Tiley and Cullen, 1992). Recently, the author demonstrated that the Rev function of the FIV required cellular factor(s) in a feline cell line (CHAPTER 4 in this thesis). This data is consistent with the case of the HIV-1 Rev protein (Trono and Baltimore, 1990; Vaishnav *et al.*, 1991; Winslow and Trono, 1993). These findings suggest that Rev proteins of the lentiviruses are important not only for virus expression but also for the virus cell tropism.

Late regulatory gene

1) *vif*

The *vif* gene of the FIV is located in the central region between *pol* and *env* genes of the genome (Fig. 1). The hydrophilic and basic natures of the *vif* product were similar to the *vif* proteins of HIV and the Q product of visna virus (Sonigo *et al.*, 1985). The mRNA of the *vif* gene is expressed by singly spliced event and contain the RRE, and its expression is regulated by the *rev* gene (CHAPTERS 1 and 3 in this thesis).

In the HIV-1, expression of the *vif* gene is also regulated by the *rev* gene (Garrett *et al.*, 1991; Schwartz *et al.*, 1991). The *vif* defective mutant of the virus

significantly reduced the cell-free virus infectivity to certain cells, and this phenotype is cell-dependent (Blanc *et al.*, 1993; Fan and Peden, 1992; Fisher *et al.*, 1987; Gabuzda *et al.*, 1992; Sakai *et al.*, 1993; Shibata *et al.*, 1990*a*, *b*; Strebel *et al.*, 1987). Recently, Sakai *et al.* (1993) reported that the Vif involved in the maturation of virus particles, because incorporation of the Env surface protein into virions of the *vif* mutant was greatly restricted.

The Vif of the FIV also affects cell-free virus infectivity (CHAPTER 1 in this thesis). The cell-free virus from cells transfected with *vif* mutant clone could not infect feline CD4 positive T-cell lines. The infectivity of the *vif* mutant, however, was demonstrated in a coculture of transfected cells and feline T-cell lines (CHAPTER 1 in this thesis). This phenotype of the *vif* mutant virus of the FIV is very similar to that of HIV, and it is likely that *vif* plays an important role in the biology of the lentivirus.

Other regulatory genes of lentiviruses

1) *nef*

In addition to Tat and Rev, primate lentiviruses encode a third early regulatory gene termed *nef*. Nef is a myristylated phosphoprotein that is associated with

cytoplasmic membrane structures and excluded from the cell nucleus (Hammes *et al.*, 1989). Unlike Tat and Rev, the *nef* gene product is not required for the virus replication in culture cells. It has been reported that expression of Nef can result in an inhibition of HIV LTR-specific gene expression and viral replication (Ahmad *et al.*, 1988; Guy *et al.*, 1990; Luciw *et al.*, 1987; Niederman *et al.*, 1989). However, these negative effects of Nef remain controversial, because other investigators have observed no effect of the Nef protein on either viral replication or gene expression (Akari *et al.*, 1992; Bachelerie *et al.*, 1990; Cheng-Mayer *et al.*, 1989; Hammes *et al.*, 1989; Kim *et al.*, 1989). Although the role of the *nef* gene product in the viral replication cycle remains unclear, it does not appear likely that Nef acts by regulating the levels of viral gene expression. Recently, in fact, it has been reported that Nef interferes with T-cell function by down-regulating CD4 molecule expression (Garcia and Miller, 1991). Furthermore, it was also reported that a functional *nef* gene product markedly enhances viral replication and pathogenicity in rhesus macaques infected with a cloned isolate of SIV from macaque monkey (Kestler *et al.*, 1991).

Although the *nef* ORF is present in only primate lentiviruses, *nef* gene-like activity has been demonstrated in a molecular clone of FIV (Miyazawa *et al.*, 1993). This

activity was demonstrated to result in a repression of the FIV LTR promoter activity. To confirm *nef* gene-like activity in the FIV genome, more detail investigations will be needed.

2) *vpu*

The Vpu protein is unique to HIV-1, not being encoded by other lentiviruses. Viruses lacking the functional Vpu protein exhibited severe defects in the assembly and release of virus particles that accumulated predominantly in the intracellular vacuoles of infected cells (Klimkait *et al.*, 1990; Terwilliger *et al.*, 1989). In addition, it has been shown that the Vpu protein can selectively degrade CD4 in the endoplasmic reticulum (Willey *et al.*, 1992*a*, *b*). Vpu also involved in the export of capsid proteins independently of both Env-gp160 and CD4, suggesting that the Vpu protein might have more than one target in the cells (Yao *et al.*, 1992).

3) *vpr*, *vpx*

The *vpr* gene is present in HIV as well as in SIV isolates from sooty mangabeys (Dewhurst *et al.*, 1990; Hirsch *et al.*, 1989), mandrills (Tsujimoto *et al.*, 1988, 1989), a chimpanzee (Huet *et al.*, 1990), and macaque species (Kestler *et al.*, 1990). The *vpx* gene is present in HIV-2 and SIV from

African green monkeys (Fukasawa *et al.*, 1988; Ohta *et al.*, 1988). These two proteins are detected in its virions, and are a structural protein (Cohen *et al.*, 1990*a*; Henderson *et al.*, 1989; Yu *et al.*, 1989, 1990). Although both the *vpr* and the *vpx* genes are not required for the virus replications, these protein can enhance the virus replication rate in cultures (Cohen *et al.*, 1990*b*; Dedra *et al.*, 1989; Guyader *et al.*, 1989; Hattori *et al.*, 1990; Hu *et al.*, 1989; Marcon *et al.*, 1991; Ogawa *et al.*, 1989; Yu *et al.*, 1991). Interestingly, behaviors of these protein defective viruses in cell cultures are exactly like those of the ORF-A mutant virus of the FIV in T-cell lines and primary cultures (CHAPTER 2 in this thesis).

Furthermore, it has been proposed that Vpr can enhance HIV-1 LTR-dependent gene expression two- to three-fold (Cohen *et al.*, 1990*b*), and this observation is also similar to the ORF-A gene of the FIV. Thus, it is important to examine whether the ORF-A product is incorporated in the virion or not.

E: CONCLUSIONS

Lentiviruses are distinguished from simple retroviruses not only on the basis of their larger coding capacity but also by a particular pattern of viral gene expression that

is dependent on the action of regulatory proteins, such as Rev (Fig. 4). Although the genomic organization, splicing manners, and regulation of gene expression of the non-primate lentiviruses including FIV are relatively simple compared with those of primate lentiviruses, current evidence suggests that proteins functionally equivalent to HIV-1 Rev, such as FIV Rev, have maintained a very similar mechanism of action. Presumably, this conservations may relate to the facts in which all lentiviruses appear to give rise to long-term, chronic infections marked by high levels of latently infected cells. In fact, it has suggested that the level of Rev activity in the infected cell may well be a primary determinant of the ability of the lentiviruses to establish a latent infection (Garcia-Blanco and Cullen, 1991; Pomerantz *et al.*, 1990, 1992). Therefore, the study of the gene expression and its regulation observed in the lentiviruses seem to be important for understanding the pathogenesis and host immune response caused by the infection.

F: LEGENDS FOR FIGURES

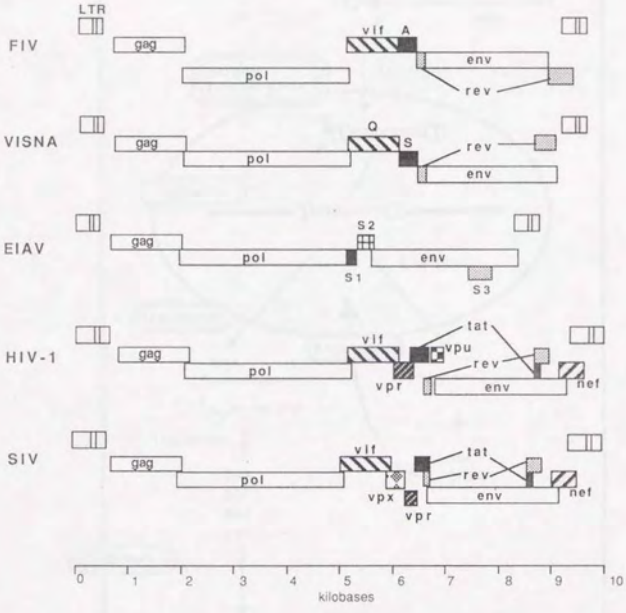
Fig. 1 Comparative genomic organization of lentiviruses. The organization and relative position of ORFs are shown. The Q ORF of visna virus is presumed to be a *vif*-like gene. The S1 and S3 ORFs in EIAV are a exon of *tat* and *rev* gene, respectively. The long terminal repeats (LTRs) contain the U3, R, and U5 regions.

Fig. 2 The FIV life cycle. The binding of FIV to cellular receptor results in the entrance of the viral core into the cells. Viral RNA is converted to DNA, enters the nucleus, and integrates into the cellular DNA. The structural proteins are synthesized and then they assemble into particles containing the viral enzymes and two copies of the genomic RNA. The particles bud from the cell surface and mature through the action of the viral protease.

Fig. 3 The organization of the LTRs of HIV, visna virus, and FIV. The location of known and potential transcriptional control region are indicated. The location of U3, R, and U5 are shown. TATA, TATA box; TAR, transactivation response element.

Fig. 4 Model for feedback regulation of lentivirus expression by virus encoding regulatory genes. Tat is a positive activator acting via the TAR element in the LTR. Rev acts as a traffic signal in the nucleus promoting the transport of RRE^+ mRNA to the cytoplasm. ORF-A gene of FIV is predicted to be a *tat*-like gene.

Fig. 1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gag
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vif
A
env
rev
VISNA
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Q
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rev
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pol
S2
S1
env
S3
HIV-1
gag
pol
vif
vpr
tat
vpu
rev
env
nef
SIV
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pol
vif
vpx
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9
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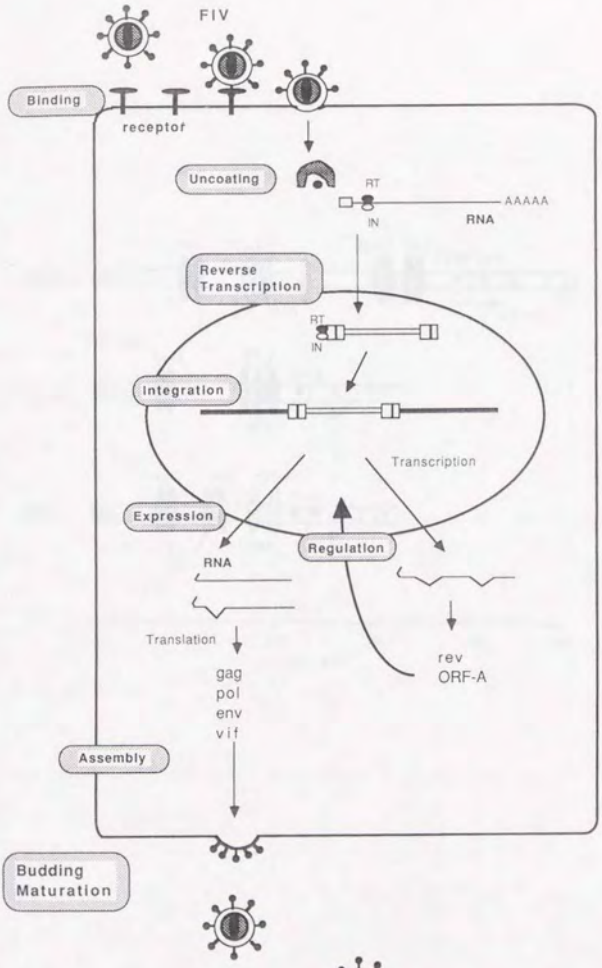
kilobases

Fig. 2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RT
IN
AAAAA
RNA
Reverse Transcription
RT
IN
Integration
Transcription
Expression
Regulation
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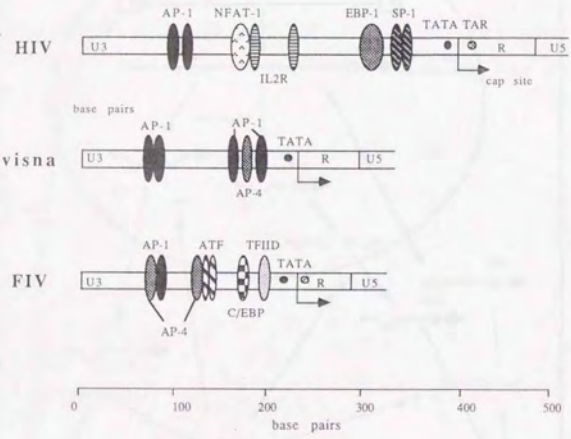
Translation
gag
pol
env
vif
rev
ORF-A
Assembly
Budding Maturation

Fig. 3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EBP-1
SP-1
TATA TAR
U3
R
U5
IL2R
cap site
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AP-1
TATA
U3
R
U5
AP-4
FIV
AP-1
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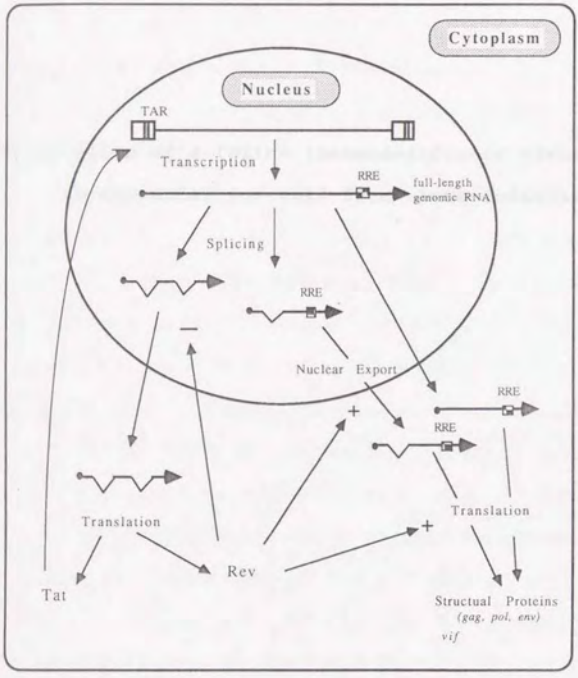
AP-4
0
100
200
300
400
500
base pairs

Fig. 4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Transcription
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Splicing
RRE
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RRE
RRE
Translation
Translation
Rev
Tat
Structual Proteins
(gag, pol, env)
vif

CHAPTER 1

Identification of a feline immunodeficiency virus gene which is essential for cell-free virus infectivity

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SUMMARY

Feline immunodeficiency virus (FIV) contains at least three small open reading frames (ORFs) in the genome, in addition to the three structural genes. Of these, two ORFs (putative *vif* and ORF-A) have unknown functions. Northern blot analysis of mRNAs from a FIV-infected cell line showed that the putative *vif*-specific mRNA was expressed as 5.2 kilobase species. We constructed mutants carrying a deletion either in the *vif*-like or *rev* genes from an infectious molecular clone of FIV to examine the function and expression of putative *vif* gene. Although the *vif* mutant produced virion-associated reverse transcriptase at a normal level upon transfection, cell-free virus prepared from the transfected cells could not infect feline $CD4^+$ cells. The infectivity of the *vif* mutant, however, was demonstrated in a coculture of transfected cells and feline $CD4^+$ cells. The *rev* minus mutant did not express some viral mRNAs including the *vif* species in the cytoplasm of transfected cells. We conclude that FIV contains the *vif* gene which is structurally and functionally similar to that of the primate lentiviruses.

INTRODUCTION

Feline immunodeficiency virus (FIV) is a lentivirus of the retrovirus family that causes a slowly progressive multiorgan disease in cats (Ishida *et al.*, 1989; Pedersen *et al.*, 1987, 1989; Yamamoto *et al.*, 1988). It is closely related in biological characters and genome organization to other members of mammalian lentivirus, including human, simian, bovine immunodeficiency viruses, equine infectious anemia virus, visna virus, and caprine arthritis-encephalitis virus (for a review, see reference Narayan and Clements, 1990). The hallmarks of lentivirus infections are virus persistence in the face of host immune responses and induction of chronic disease. Lentiviruses have several extra open reading frames (ORFs) in their genome, in addition to the three structural genes (*gag*, *pol*, and *env*) in common with other retroviruses. Human immunodeficiency virus type 1 (HIV-1) genome consists of six regulatory genes and their functions have been studied extensively by molecular biological analyses. Of these, two trans-activator genes, *tat* and *rev*, are essential for virus gene expression (Dayton *et al.*, 1986; Fisher *et al.*, 1986; Sodroski *et al.*, 1985; Terwilliger *et al.*, 1988). *Nef* is not essential for the replication in tissue cultures (Luciw *et al.*, 1987), although it may have important functions in vivo (Kestler *et*

al., 1991). *Vif*, *vpr*, and *vpu* also influence the replication of the virus (Fisher *et al.*, 1987; Ogawa *et al.*, 1989; Strebel *et al.*, 1987, 1988). The *vpx* gene, which is present in the genomes of HIV-2 and simian immunodeficiency viruses (SIVs), is necessary for the viruses to grow in peripheral blood lymphocytes (Guyader *et al.*, 1989). The expression pattern of viral mRNAs was examined in HIV/SIV and visna virus systems (Schwartz *et al.*, 1990*a*; Viglianti *et al.*, 1990; Vigne *et al.*, 1987). The small, multiply-spliced mRNA species, encoding the viral regulatory proteins Tat and Rev, are expressed in the early stage of infection. The HIV-1 Rev activates the expression of late genes including the three viral structural genes (*gag*, *pol*, and *env*), and *vif* and *vpr* genes, whose mRNAs contain the *cis*-acting RNA target sequence for Rev designated as *rev*-responsive element (RRE) (Garrett *et al.*, 1991; Schwartz *et al.*, 1991).

Sequence analyses of several FIV strains showed that the genome contains at least three small ORFs, which may encode viral regulatory proteins (Maki *et al.*, 1992; Olmsted *et al.*, 1989*a*, *b*; Talbott *et al.*, 1989). Two ORFs, which are located in the region between *pol* and *env*, are called *vif* (a tentative name) or ORF 1, and ORF-A or ORF 2. Putative *vif* is similar in size and location to the *vif* gene of primate lentivirus (Chakrabarti *et al.*, 1987; Fukasawa *et al.*, 1988; Guyader *et al.*,1987; Maki *et al.*, 1992; Talbott *et al.*,

1989). ORF-A resembled the first exon of *tat* by its size and location in the FIV genome (Chakrabarti *et al.*, 1987; Fukasawa *et al.*, 1988; Guyader *et al.*, Ratner *et al.*, 1985). A short 3' ORF that overlaps the long terminal repeat (LTR) is called ORF-B or ORF 4 (Maki *et al.*, 1992; Olmsted *et al.*, 1989*a, b*). Recently, we have shown by transfection analysis that ORF-B of FIV is at least a part of *rev* gene (Kiyomasu *et al.*, 1991). In this chapter, we report biochemical and biological analyses of the putative *vif* gene product of FIV. Like the *vif* genes of various primate lentiviruses, this ORF is essential for cell-free viral infection, and its expression is activated by the Rev.

Cell culture and DNA transfection. A feline $CD4^+$ lymphoblastoid cell line (MYA-1 cells) (Miyazawa *et al.*, 1989*b*) was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Crandell feline kidney (CRFK) cells (ATCC CCL94) were maintained in Dulbecco modified Eagle's medium supplemented with 10% heat-inactivated FCS. Feline peripheral blood lymphocytes (fPBLs) were prepared and cultured as described previously (Miyazawa *et al.*, 1989*b*). For transfection, uncleaved plasmid DNA was introduced into CRFK cells by the calcium phosphate coprecipitation method (Graham *et al.*, 1973; Wigler *et al.*, 1979).

Infection. The infectivity of progeny virions produced in transfected CRFK cells was assayed in MYA-1 cells and fPBLs. Culture supernatants from the transfected cells were filtered (pore size, 0.45μm), and appropriate volumes were added to 10^6 cells and incubated at 37°C. After 1 h, the mixture was washed twice in fresh and was incubated at 5% CO_2 and 37°C as previously described (Folks *et al.*, 1985). When needed, transfected cells were cocultivated with MYA-1 cells to amplify any progeny virions produced as previously described (Adachi *et al.*, 1986).

RT assays. Virion-associated reverse transcriptase (RT) activity was measured as described previously (Willey *et al.*, 1988). Briefly, 10 μl of culture supernatant was mixed with 50 μl of an RT reaction mixture containing [^{32}P]dTTP. After 90 min at 37℃, 10 μl of the mixture was spotted onto DE81 paper, washed four times in 2X SSC and spots were visualized by autoradiography.

RNA extraction and Northern (RNA) blotting. Total cellular RNA was extracted from MYA-1 cells infected with FIV TM2 strain (Miyazawa *et al.*, 1989*a*) by the guanidine thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Cytoplasmic RNA was prepared from transfected CRFK cells as described before (Sambrook *et al.*, 1989). Poly(A)$^+$ RNA was enriched from the total RNA and from the cytoplasmic RNA by using Oligotex-dT30 (Takara Shuzo Co., Ltd., Kyoto, Japan). RNA was electrophoresed through 1% agarose gel containing 2.2M formaldehyde, and analyzed by Northern blot hybridization as previously described (Rabson *et al.*, 1985). Cut-out DNA fragments encompassing most of the 5' LTR (nucleotide (nt) number between 16 and 515 of TM2 strain), putative *vif* region (nt between 5290 and 5936), and *env* region (nt between 6694 and 7110), were labeled with ^{32}P by the random primed DNA labeling system (Boehringer

Mannheim Yamanouchi Co., Ltd., Tokyo, Japan) and used as probes (Fig. 1-1A).

Western blotting. Lysates of transfected CRFK cells were prepared as previously described (Willey *et al.*, 1988), and proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes. The membranes were incubated overnight at room temperature with serum from a cat infected with FIV, incubated with ^{125}I-protein A for 3 h, washed, and then visualized by autoradiography (Willey *et al.*, 1988).

DNA constructs. The molecular cloning of unintegrated virus DNA of FIV TM2 strain and complete nucleotide sequence of the virus have been described (Maki *et al.*, 1992) (GenBank accession number M59418). Mutant clones constructed in vitro were derived from an infectious molecular clone of FIV, designated pTM219 (Kiyomasu *et al.*, 1991). To construct a mutant with a deletion in the putative *vif*, termed pTM-Ac, a fragment encompassing *vif* region was first subcloned into pUC19, sequence between the two *Acc*I sites (nt 5300 and 5328) was deleted, and then the mutated fragment was put back into pTM219. A *rev* mutant, pTM-Nd has been described (Kiyomasu *et al.*, 1991).

cDNA synthesis and PCR amplification. The polymerase chain reaction (PCR) (Saiki *et al.*, 1988) was used to determine the splice donor and splice acceptor sites of FIV TM2 strain in infected MYA-1 cells. Poly(A)$^+$ RNA (0.5 to 1 μ g) was reverse-transcribed in the presence of the antisense oligonucleotide primer; PRVIA, 5'-CAGGTAGACTCGATATGTAC-3' (nt 5335 to 5316 of TM2), and the DNA was amplified in the same tube as follows. The RNA was heated at 70℃ for 5 min and cooled on ice. First-strand synthesis of cDNA was carried out in a total volume of 20μ l containing 75mM KCl, 50mM Tris-HCl PH8.3, 5mM $MgCl_2$, 0.01M DTT, 10mM each dNTP, 1U of RNasin (Promega Co., Madison, U.S.A), 10U of Molony murine leukemia virus reverse transcriptase (Gibco. BRL. Life Thechnologies Inc., Gaithersburg, U.S.A), and 0.5μ g of antisense oligonucleotide primer. The reaction mixture was incubated at 45℃ for 2 h. PCR amplification of single-stranded DNA was carried out after addition of 0.5μ g of sense oligonucleotide primer; PRLTS, 5'-GAACTCCTGCAGACCTTGTG-3' (nt 436 to 455 of TM2), and 1U of *Thermus aquaticus* DNA polymerase (Saiki *et al.*, 1988) (Taq polymerase; Perkin-Elmer Cetus, Norwark, U.S.A). The reaction mixture was overlaid with 1 drop of mineral oil (Sigma Chemical Co., St.Louis, U.S.A) and incubated at 94, 55, and 72℃ for 1, 2, and 3 min, respectively. The cycle was repeated 30 times

in a thermal cyclic reactor (Hoei-Science Co., Tokyo, Japan).

Cloning and sequence of amplified cDNA. After amplification of the cDNA by PCR, the product was digested with *Sac*I and *Taq*I, and cloned into pUC119 vector digested with *Sac*I and *Acc*I. For sequencing, the double-stranded plasmid DNA was denatured and dissolved in a reaction mixture together with the appropriate sequencing primer. The DNA sequences were determined by the Sequenase version 2.0 kit (United States Biochemical Co., Cleveland, U.S.A).

Expression of *vif*-like ORF of FIV

There was a potential *vif* gene in the FIV genome as predicted by its location and size. The putative *vif* of FIV TM2 strain consisted of 789 nucleotides and located in a central region (Fig. 1-1A). A theoretical translation product of the ORF was 251 amino acid long (Fig. 1-1B) and its molecular weight was calculated to be approximately 29 kilodalton. The hydrophilic and basic (13.5% Arg + Lys) natures of the product was also similar to the *vif* proteins of HIV/SIV and the ORF Q product of visna virus (Sonigo *et al.*, 1985). There was a homology among the predicted amino acid sequences of the putative *vif* protein of FIV, the ORF Q of visna virus, and *vif* of HIV-1 and SIV_{MAC}. Especially, it is interesting to note that a cysteine-rich region of FIV was significantly conserved among the four distinct lentiviruses (Fig. 1-1C).

To determine whether this *vif*-like ORF of FIV is actually transcribed, poly(A)$^+$ RNA was isolated from MYA-1 cells infected with FIV TM2 strain, and examined by Northern blot hybridization (Fig. 1-2) using three specific probes (Fig. 1-1A). The LTR probe, which was expected to hybridize with all viral mRNAs, detected at least four viral-specific mRNAs, 9.2, 5.2, 4.4, and 1.7 kilobases (kb) (Fig. 1-2, lane

b). The *vif* probe detected 9.2 and 5.2 kb but not smaller mRNA species (Fig. 1-2, lane d). By the *env* probe, 9.2, 5.2 and 4.4 kb RNA species were detected (Fig. 1-2, lane f). These RNAs were similar in size to those of viral RNA species detected in HIV-1- or visna virus-infected cells (Feinberg *et al.*, 1986; Vigne *et al.*, 1987). Thus, the 9.2 kb mRNA might represent the full-length genomic mRNA (*gag-pol* mRNA). The small mRNAs (around 1.7 kb), which were not hybridized with the *vif* and *env* probes, were predicted to be multiply-spliced mRNA species. The medium size mRNAs, the 5.2 and 4.4 kb species, would represent *vif* and *env* mRNAs, respectively.

To determine the splice donor and acceptor sites for the *vif* ORF, partial cDNAs were synthesized from poly(A)$^+$ RNA of FIV TM2 strain-infected cells, and subjected to PCR amplification (Fig. 1-3). After amplification, only single band was detected by electrophoresis (data not shown). The sequence analysis of the product (3 clones were sequenced) indicated that the 5' splice donor site was found at base 611 of the TM2 sequence (Fig. 1-3), and that the splice acceptor site for the *vif* ORF was located at base 5194 (48 bases 5' to the first AUG of the *vif* ORF) (Fig. 1-3). Together with the results of Northern blot hybridization, it was highly likely that a major *vif* mRNA utilized this splice donor and acceptor, and continued to the poly(A) site at the

3' end of genome (Fig. 1-3). Furthermore, we determined that this splice donor site was a common splice donor for all subgenomic viral RNAs and that this splice acceptor was also utilized for one of the ORF-A mRNA species which was multiply-spliced (Tomonaga *et al.*, 1993*c*. CHAPTER 3 in this thesis).

Mutational analysis of *vif*-like ORF of FIV

To determine the functional role of the putative *vif*, a deletion mutant was constructed, and characterized biologically. The structure of the mutant pTM-Ac, which lacks the sequence between two *Acc*I sites (Fig. 1-4A), was confirmed by sequencing. Mutants pTM-Ac and pTM-Nd (Kiyomasu *et al.*, 1991) were evaluated for transient expression of RT and *gag* proteins in CRFK cells. RT production was observed in the CRFK cells transfected with pTM219 (wild type) and pTM-Ac (putative *vif* mutant) but not with pTM-Nd (*rev* mutant) (Fig. 1-4B). In addition, four *gag* proteins could be found in the lysates prepared from CRFK cells transfected with pTM219 and pTM-Ac, whereas no proteins were found in those of the cells transfected with pTM-Nd (no significant difference from negative control) (Fig. 1-4C).

Growth ability of progenies produced in the transfected cells was then tested in MYA-1 cells. As judged by RT production, no evidence of infectivity was obtained with

cell-free virus obtained from pTM-Ac (Fig. 1-5A). Viable cell counts of MYA-1 cells confirmed the lack of productive infection by the TM-Ac virus (Fig. 1-5B). Results obtained with cell-free TM-Ac virus in fPBLs were similar to those obtained in MYA-1 cells (data not shown). However, when the coculture method was used, progeny production of the TM-Ac was observed (Fig. 1-6), as was the case for HIV-1 and HIV-2 *vif* mutant strains (Sibata *et al.*, 1990*a*; Strebel *et al.*, 1987).

We next examined the Rev-dependency of the 5.2 kb RNA by Northern blotting of cytoplasmic poly(A)$^+$ RNA. When the *vif* specific probe was used, 9.2 and 5.2 kb hybridization bands, which were full-length genomic and *vif* mRNAs, respectively, were detected in the cells transfected with pTM219 (Fig. 1-7, lane a). However, the probe failed to detect any specific mRNAs in the cells transfected with pTM-Nd (Fig. 1-7, lane b). In contrast, a probe specific for LTR detected a large amount of the small, multiply-spliced mRNA species in the cells transfected with pTM-Nd more than with pTM219 (Fig. 1-7 lane e). These results indicated that the cytoplasmic expression of the 9.2 kb (*gag-pol* mRNA) and 5.2 kb (*vif* mRNA) RNAs was activated by Rev, as was the case for HIV-1 (Garrett *et al.*, 1991; Schwartz *et al.*, 1991).

DISCUSSION

The purpose of this study was to determine whether FIV has a *vif* gene. All the data presented here regarding structural (Figs. 1-1, 1-2, 1-3) and functional analyses (Figs. 1-4, 1-5, 1-6, 1-7) of the *vif*-like ORF strongly suggested that FIV carries the *vif* gene.

Comparison of predicted amino acid sequences of *vif* and ORF Q proteins of the different lentiviruses showed that a cysteine-rich region of FIV was particularly well-conserved (Fig. 1-1C). The Northern blot analysis presented in Fig. 1-2 indicated that the *vif* ORF in FIV genome was certainly expressed as 5.2 kb RNA in the infected cells. The *vif* mRNA was produced through a singly-splicing event from genomic RNA (Fig. 1-3), and its cytoplasmic expression was Rev-dependent (Fig. 1-7).

The *vif* mutant pTM-Ac produced RT and *gag* proteins at a normal level in transient transfection experiments (Fig. 1-4B, C). When cell-free virus stock was used as inocula, however, the TM-Ac virus was not infectious (Fig. 1-5A, B) as reported for the *vif* mutants of HIV-1 and HIV-2 (Fisher *et al.*, 1987; Shibata *et al.*, 1990*a*; Strebel *et al.*, 1987). The infectivity of the *vif* mutant of FIV could be demonstrated by the cocultivation procedure (Fig. 1-6), a common biological property of lentiviral *vif* mutants. From a

virological point of view, it is interesting that *vif* activity appears to be conserved in FIV and HIV. Until to date, only the *rev* gene has been demonstrated to be present among various regulatory genes of the lentiviruses including FIV. Although the molecular basis for the activity of *vif* is not known yet, its wide distribution is biologically important. It is likely that the *vif* plays one of the key roles for the biology of lentiviruses.

LEGENDS FOR FIGURES

Fig. 1-1 Structure of the putative *vif* of FIV.

(A) A schematic representation of FIV genome organization. LTRs and open reading frames (ORFs) of the clone are based on the nucleotide sequence of TM2 strain (Maki *et al.*, 1992). Solid bars indicate the location of the probes used for hybridization (Fig. 1-2 and Fig. 1-7). The exact positions of the probes are described in Materials and Methods. (B) Predicted amino acid sequence of putative *vif* gene product of FIV. The cysteine-rich region of FIV putative *vif* is underlined. (C) Partial Alignment of the amino acid sequence of predicted *vif* of FIV, HIV-1, and SIV$_{MAC}$ and ORF Q of visna virus (VISNA). Asterisks indicate the cysteine residues in putative *vif* of FIV. Gaps have been introduced to align all the related proteins. Residues considered to be homologous are boxed. Sequence of putative *vif* of FIV TM2 (residue 181-226) was compared with that of ORF Q product of VISNA (residue 153-198), and those of *vif* of HIV-1 432 (residue 129-170) and SIV$_{MAC}$ 239 (residue 130-172). Sequence data of HIV-1 432 strain (GenBank accession number M19921), SIV$_{MAC}$ 239 strain (GenBank accession number M33262), VISNA (Sonigo *et al.*, 1985) were used.

Fig. 1-2 Northern blot analysis of poly(A)$^+$ RNA from MYA-1 cells infected with FIV TM2 strain. Poly(A)$^+$ RNA was prepared from mock-infected cells (lanes a, c, e), TM2-infected cells (lanes b, d, f). The hybridization probes used are indicated on the top of the lane (see Fig. 1A). Locations of the 28S and 18S rRNAs are indicated on the right. kb, kilobases.

Fig. 1-3 Identification of the splice donor and splice acceptor sites utilized for the 5.2 kb RNA (*vif* mRNA) of FIV TM2 isolate. FIV genome organization is shown at the top. Locations of sense and antisense PCR primer are indicated by arrows (see MATERIALS AND METHODS). Solid bars indicate exon present in the *vif* mRNA. Nucleotide positions of splice donor and acceptor are indicated. SD, splice donor; SA, splice acceptor.

Fig. 1-4 Construction and characterization of proviral FIV mutants. The locations of mutations (deletions) and enzymes used to construct mutants are shown at the top. (A) Mutant designations. (B) RT production in CRFK cells 48 h after transfection. Cells were transfected with pTM219 (wild type) (219), pTM-Ac (*vif* mutant) (Ac), pTM-Nd (*rev* mutant) (Nd), and pUC19 (negative control) (Cr). (C) Detection of *gag* proteins produced in transfected CRFK cells by Western blot

analysis with serum from a FIV-infected cat. Cell lysates were prepared from the cells transfected with pTM219 (219), pTM-Ac (Ac), pTM-Nd (Nd), and pUC19 (Cr).

Fig. 1-5 Growth kinetics in MYA-1 cells by a *vif* mutant virus. (A) RT production in MYA-1 cells. Equivalent amounts (1×10^6 cpm of RT activity) of cell-free pTM219 (219), and pTM-Ac (Ac) virus from transfected CRFK cells were added to 10^6 cells, and RT production was monitored at the indicated intervals. Values represent the day after infection. (B) Viable cell counts of MYA-1 cells infected with the virus clones. Viable cell numbers of infected MYA-1 cells were determined by trypan blue staining at intervals. Symbols: open circles, mock-infected; closed circles, TM219-infected; closed triangles, TM-Ac-infected.

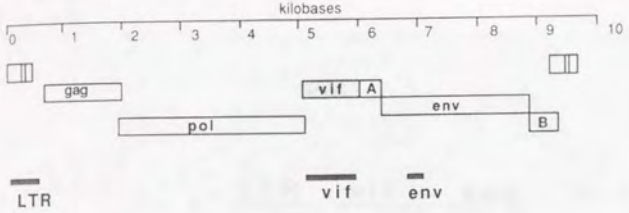
Fig. 1-6 Kinetics of viral RT production in the coculture assay. RT expression in transfected CRFK cells cocultured with MYA-1 cells is shown. At 48 h post-transfection, CRFK and MYA-1 cells were mixed, and maintained in RPMI 1640 medium for monitoring the virus growth. Values above the autoradiograms indicate the day after coculture. Symbols: 219+, CRFK cells were transfected with pTM219 and cocultured; Ac+, CRFK cells were transfected with pTM-Ac and cocultured; Ac-, CRFK cells were transfected with pTM-Ac but

did not coculture with MYA-1 cells and served as a control; mock, mock-transfected CRFK cells were cocultured with MYA-1 cells as a negative control.

Fig. 1-7 Cytoplasmic expression of FIV RNA in the transfected cells. CRFK cells were transfected with pTM219 (lanes a, d), pTM-Nd (lanes b, e), or pUC19 (lanes c, f). Cytoplasmic RNA was isolated 72 h after transfection, and Northern blot analysis was performed either with the *vif* or the 5' LTR probe (see Fig. 1-1A). kb, kilobases.

Fig. 1-1

A



B

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        70          80          90         100         110         120
EGIRWSFHTR DYYIGYVREM VAGSSLPDSL RLYVYISNPL WHQSYRPGLT NFNTEWPFVN
       130         140         150         160         170         180
MWIKTGFMWD DIESQNICKG GEISHGWGPG MVGIVIKAFS CGERKIKITP VMIIRGEIDP
       190         200         210         220         230         240
TEWCGDCWNL MCLKYSPPNT LQRLAMLACG KEAKEWRGCC NQRFVSPFRT PCDLEVVQNK
       250
PKRNLLWTGE L
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C

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                *   *       *                   *                   *   *
FIV     TEWCGDCWNL MCLKYSPPNT LQRLAMLACG KEAKEWR--GC CNQRFVS
VISNA   WDLCKSCIQG EIVKNTNPRS LQRLALLALA KD-HVFQVMPL W--RARR
HIV-1   SPRC-E-YQA GHNKV--G-S LQYLALAALI KP-KQIK-PPL PSVRKLT
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Fig. 1-2

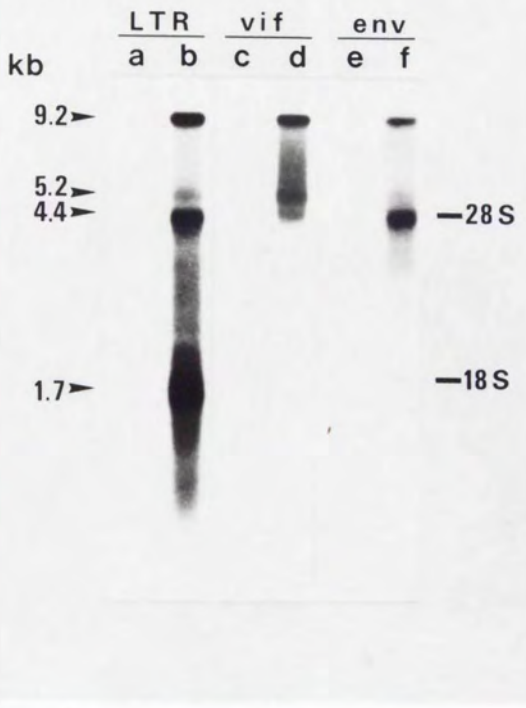
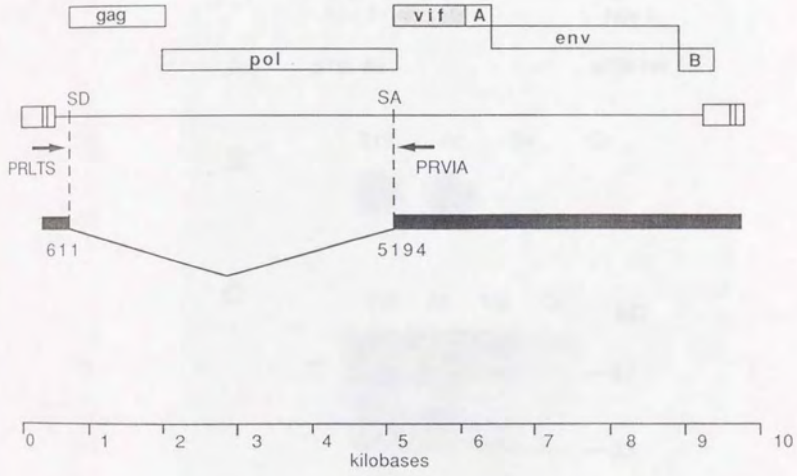


Fig. 1-3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8
9
10
kilobases

Fig. 1-4

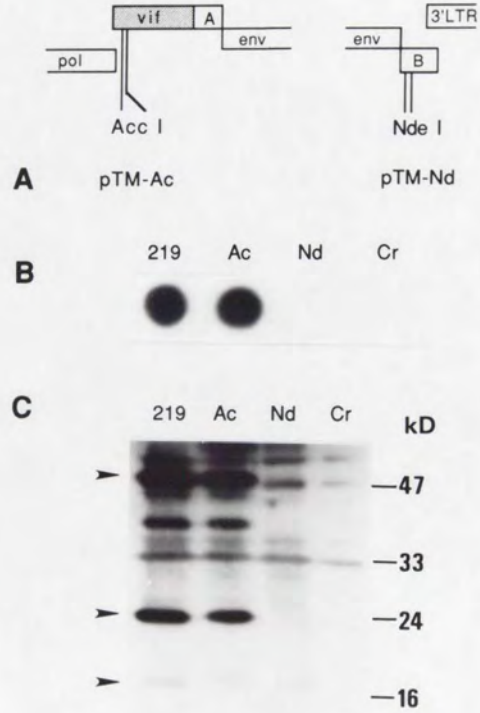

vif
A
env
pol
3'LTR
env
B
Acc I
Nde I
A
pTM-Ac
pTM-Nd
B
219
Ac
Nd
Cr
C
219
Ac
Nd
Cr
kD
47
33
24
16

Fig. 1-5

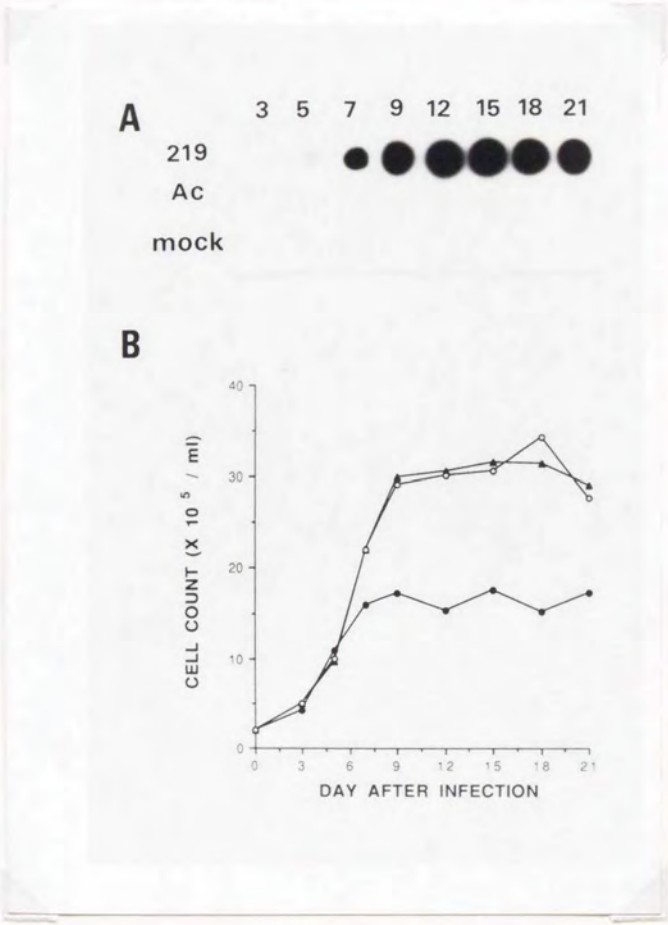

A
3 5 7 9 12 15 18 21
219
Ac
mock
B
CELL COUNT (X 10 5 / ml)
40
30
20
10
0
0 3 6 9 12 15 18 21
DAY AFTER INFECTION

Fig. 1-6

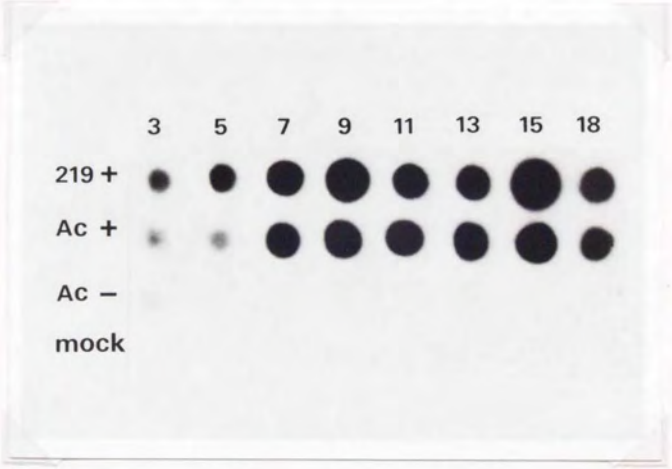
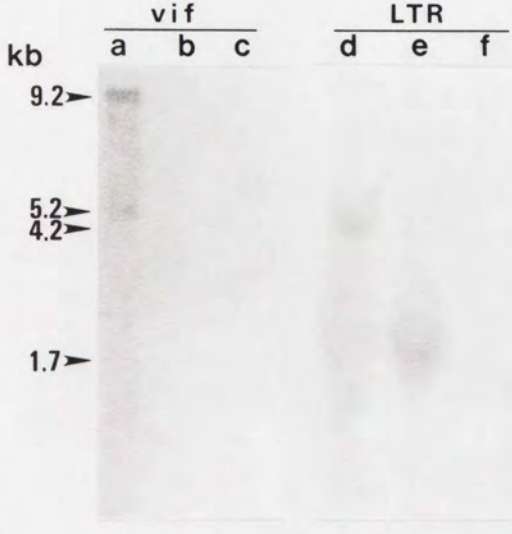


Fig. 1-7



CHAPTER 2

The feline immunodeficiency virus ORF-A gene facilitates efficient viral replication in established T-cell lines and peripheral blood lymphocytes

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SUMMARY

Frameshift mutants corresponding to all of the identified ORFs, including ORF-A gene which has unknown function, of feline immunodeficiency virus were constructed in vitro. Upon transfection into cells, no significant difference between phenotypes of ORF-A mutant clone and those of wild type clone was demonstrated. Although only ORF-A mutant virus among all mutant viruses from transfected cells showed infectivity in established T cell lines, the replication and propagation of the ORF-A mutant virus were efficiently reduced when compared with those of the wild type virus. Moreover, loss of the function of ORF-A gene result in a severe defect in the productive infection in primary peripheral blood lymphocytes both in the amount of reverse transcriptase activity produced and in core protein expression. These findings demonstrate that the ORF-A gene of FIV is required for efficient viral replication and also suggest that the ORF-A gene is likely to be important for natural infection.

INTRODUCTION

Feline immunodeficiency virus (FIV) belonging to a lentivirus group of the retrovirus family, is closely related in cell tropism and genome organization to human immunodeficiency virus, simian immunodeficiency virus, and the other ungulate lentiviruses (Pedersen *et al.*, 1987; Yamamoto *et al.*, 1988). It is known that the FIV is cytopathic in tissue culture, and has a tropism for $CD4^+$ subset of T cells, macrophages, and other cells (Brown *et al.*, 1991; Brunner *et al.*, 1989; Kawaguchi *et al.*, 1992*a*; Toyosaki *et al.*, 1993). The genetic organization of the FIV is relatively simple compared with that of primate lentiviruses in that the gene contains at least three short open reading frames (ORFs), *vif*, ORF-A (or ORF 2), and *rev*, in addition to the structural genes common to all retroviruses (Maki *et al.*, 1992; Olmsted *et al.*, 1989*a*, *b*; Talbott *et al.*, 1989). Recently, the activity or expression of a *trans*-activator gene, *rev*, has been shown extensively by molecular biological analyses (Kiyomasu *et al.*, 1991; Phillips *et al.*, 1992), and the location of the target sequence for Rev, Rev-responsive element (RRE), was also reported at the 3' end of the *env* ORF (Phillips *et al.*, 1992). Another non-structural gene, *vif*, is expressed as a 5.2-kb mRNA by single-splice event and is necessary for

cell-free virus infection in feline T lymphoblastoid cells (Tomonaga *et al.*, 1992, CHAPTER 1 in this thesis).

In contrast, only a few information on the ORF-A gene have been showed in previous studies; (i) the mRNAs which encode ORF-A sequences are detected in FIV-infected cells, as doubly- or triply-spliced transcripts (Phillips *et al.*, 1992; Tomonaga *et al.*, 1993*c*, CHAPTER 3 in this thesis), (ii) A molecular clone of FIV, Petaluma isolate (clone 34TF10), which has a premature stop codon in the middle of the ORF-A (ORF 2) sequence, is infectious, and this shortened ORF-A does not appear to have a role in determining host cell range (Phillips *et al.*, 1990), and (iii) ORF-A expression plasmid of PPR clone can activate very weakly LTR of 34TF10 clone in CRFK cells but not activate own LTR (Sparger *et al.*, 1992). Functional analysis of ORF-A gene, however, has not yet been carried out. Furthermore, it is unclear the effects of the ORF-A gene on replication and propagation of the virus. In this chapter, as one approach towards understanding the role of the ORF-A gene in the complex life cycle and cell tropism of FIV, we have constructed mutants corresponding to all of the identified ORFs, including ORF-A, from an infectious DNA clone of FIV. Although the ORF-A mutant virus can infect to established $CD4^+$ T cell lines, efficiency of the viral replication is reduced compared with wild type virus. The

mutant displays, moreover, a severe defect in the productive infection in primary peripheral blood lymphocytes (PBLs). Our finding suggests that the ORF-A gene enhances efficient viral replication and plays an important role in the in vivo life cycle of the virus.

Cell cultures and DNA transfection. Two feline T lymphoblastoid cell lines, MYA-1 (Miyazawa *et al.*, 1989*b*) and FeL-039 cells (Tokunaga *et al.*, 1992), which were positive for feline CD4 and negative for feline CD8, were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Crandell feline kidney (CRFK) cells (ATCC CCL94) were maintained as described in CHAPTER 1. Feline PBLs were prepared and cultured as described previously (Miyazawa *et al.*, 1989*b*). For transfection, uncleaved plasmid DNA was introduced into CRFK cells by calcium coprecipitation method as discribed in CHAPTER 1.

Infection. The infectivity of progeny virions produced in transfected CRFK cells was assayed in MYA-1 cells, FeL-039 cells, or feline PBLs. Culture supernatants from the transfected cells were filtered (pore size, 0.45 μ m), and appropriate volumes were added to 10^6 cells as previously described in CHAPTER 1.

RT assays. Virion-associated reverse transcriptase (RT) activity on cell-free medium samples was measured as described in CHAPTER 1. For quantitation, spots on DE81

paper were cut out, and RT activity was determined by scintillation counting. Samples for intracellular RT activity were prepared as follows. Infected-cell suspensions (1 ml) were pelleted and suspended in 0.5 ml of phosphate-buffered saline (PBS). Cells were disrupted in by three cycles of freezing and thawing. After vortexing and sedimentation of cell debris, 10 μ l samples of supernatant were subjected to RT assay.

Labeling and immunoprecipitation. At 48 h post-transfection, the cells were labeled for 18 h in methionine-free Eagle's medium with 150 μ Ci/ml [^{35}S]methionine at 37 ℃ and 5% CO_2. After labeling, the cells were suspended in RIPA buffer (0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.05 M Tris-HCl [pH 7.2], 0.1 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and centrifuged. The soluble fractions were immunoprecipitated with serum from an FIV-infected cat and electrophoresed through sodium dodecyl sulfate (SDS)-polyacrylamide gel as described previously (Ikuta *et al.*, 1989).

Western immunoblotting. Lysates of virus-infected cells were prepared as described previously (Tomonaga *et al.*, 1993*a*), and proteins were resolved on 12% SDS-polyacrylamide gels, followed by electrophoretic transfer

onto nitrocellulose membranes. The membranes were incubated with serum from the FIV-infected cat and with goat anti-cat IgG conjugated with horseradish peroxidase at room temperature. The peroxidase activity was detected by enhanced chemiluminescence Western blotting detection system (ECL; Amersham, Buckingghamshire, UK).

DNA construct. The infectious molecular clone, pTM219 (Kiyomasu *et al.*, 1991), was used as wild type DNA. All mutants were constructed from pTM219 by recombinant DNA techniques. Frameshift mutations were introduced into the sites (see Fig. 2-1A) by cleaving plasmid DNAs with the restriction enzymes, blunt ended by T4 DNA polymerase, and resealed by T4 DNA ligase. When necessary, the appropriate DNA fragment was first subcloned into cloning vector, the mutation was introduced, and the mutated DNA was put back into pTM219. Thus, pTM-Bs contains a 2-base-pair (bp) deletion at *Bsm*I site (nucleotide [nt] 1398 of strain TM2); pTM-Av, a 4-bp deletion at *Ava*III site (nt 2674); pTM-Bt, a 5-bp insertion at *Bst*EII site (nt 4837); pTM-Ac2, a 2-bp insertion at *Acc*I site (nt 6086); pTM-Sp, a 4-bp deletion at *Sph*I site (nt 7957). Mutant pTM-EcV was generated by cleaving pTM219 at the unique *Eco*RV site (nt 6591), insertion 8 bp of *Xho*I linker (Takara Shuzo Co. Ltd., Kyoto, Japan), and ligased by T4 DNA ligase. Constructs of

two deletion mutants, pTM-Ac and pTM-Nd, were described elsewhere (Tomonaga *et al.*, 1992, CHAPTER 1 in this thesis). Mutations within the DNA constructs were confirmed by sequencing.

PCR and DNA analysis. To analyze viral DNAs in the infected cells, genomic or extrachlomosomal DNAs were extracted from infected cells (Hirt, 1967). The DNA solution was treated with RNase A (Boehringer Mannheim Yamanouchi Co., Ltd., Tokyo, Japan), and polymerase chain reaction (PCR) technique (Saiki *et al.*, 1988) was used to amplify a central region of the genome containing the ORF-A gene with sense and antisense primers: PRVIS; 5'-GTACATATCGAGTCTACCTG-3' (nt 5316 to 5335 of strain TM2), and PRENA; 5'-TGAAGTAATGCCTGGTACCC-3' (nt 6421 to 6402). The reaction mixtures for amplification were incubated at 94, 55, and 72 ℃ for 1, 2, and 3 min, respectively. The cycle was repeated 30 times in a thermal cyclic reactor (Hoei-Science CO., Tokyo, Japan) as described in CHAPTER 1. After amplification, the products were analyzed on 1.5% agarose gel or 4% polyacrylamide gel, and visualized by UV fluorescence after staining with ethidium bromide. To denature DNA before blotting, gel was soaked in 0.5 M NaOH-0.6 M NaCl for 10 min, washed twice with H_2O, and neutralized in 2.0 M Tris-HCl (pH 7.0)- 0.6 M NaCl for 10

min. The DNA was transferred to nylon membrane. After cross-linking of DNA, the membrane was analyzed by Southern blotting hybridization. ^{32}P-labeled *Dra*III (nt 5936)-*Pst*I (nt 6263) fragment was produced by the random primed DNA labeling system (Boehringer Mannheim Yamanouchi Co., Ltd., Tokyo, Japan) and used as a probe.

Nucleotide sequence accession number. The complete nucleotide sequence data of FIV strain TM2 (Maki *et al.*, 1992) are from GenBank data base under accession number discribed in CHAPTER 1.

Biological characterization of mutant clones.

On the basis of the nucleotide sequence of FIV strain TM2 (Maki *et al.*, 1992), the mutations were introduced into the restriction sites indicated in Fig. 2-1A. The biological activity of the mutants was assessed for transient expressions of RT in supernatant and protein syntheses following transfection in CRFK cells. The results in the cells transfected with *vif* (pTM-Ac) and *rev* (pTM-Nd) mutants were described in CHAPTER 1, and used as controls to evaluate the efficiency of our system. RT production was observed in the CRFK cells transfected with *vif*, ORF-A (pTM-Ac2), or two *env* (pTM-EcV , pTM-Sp) mutants at the similar level of RT production in wild type (Fig. 2-1C and Table 2-1). In contrast, *gag* (pTM-Bs), two *pol* (pTM-Av, pTM-Bt), and *rev* mutants did not produce RT at all (Fig. 2-1C and Table 2-1).

We next analyzed the synthesis of virus-specific proteins in transfected CRFK cells. Two days after transfection, cells were labeled and examined by immunoprecipitation (Fig. 2-2). The serum used to detect virus protein mainly recognizes *gag* and *env* proteins of FIV. Two *pol*, *vif*, and ORF-A mutants directed the synthesis of virus protein comparably to wild type (Fig. 2-2, lanes c, d,

e, and f). In the cells transfected with *gag* mutant pTM-Bs, no capsid protein (CA) was found, but matrix protein (MA) could be detected (Fig. 2-2, lane b). This is because the mutation in *gag* gene was introduced in CA region (Fig. 2-1A). Although mature *env* products could not be found in both *env* mutants (Fig. 2-2, lanes g, and h), a truncated form of surface glycoprotein (SU) (Fig. 2-2, lane h, arrow) was seen in pTM-Sp transfected cells.

Since FIV strain TM2 can not display productive infection in CRFK cells (Miyazawa *et al.*, 1989*b*), this transfection assay only measured the ability of the virus to form particles containing RT that are released into the medium. Thus, the infectivity of cell-free supernatants generated in CRFK cells transfected with various DNAs was tested in MYA-1 cells. Viral infectivity was measured by RT production and also by cytopathic effect (CPE) induction. CPEs caused by the viruses were monitored daily by microscopic observation and trypan blue staining. During our observation period (30 days after infection), neither RT production nor CPE induction was noted in MYA-1 cells infected with cell-free samples obtained from all mutants except for ORF-A mutant (Fig. 2-3, and Table. 1).

Biological property of ORF-A mutant in established cell lines

The results from transient transfection assay on CRFK cells could not demonstrate any significant difference between the ORF-A mutant clone and wild type clone. In the results of experiment in Figure 2-3, however, it seemed that the ORF-A mutant virus (TM-Ac2 virus) replicates slightly slower than the wild type virus (TM219 virus). To confirm this phenotype of the virus, the biological property of TM-Ac2 virus was analyzed in more detail. First, replication of cell-free TM-Ac2 virus was observed carefully in two different T lymphoblastoid cell lines, MYA-1 cells and FeL-039 cells. An equal number of RT units (10^5 cpm) from the supernatants of the cells transfected with pTM219 or pTM-Ac2 were adjusted and used for infection. The cell cultures were monitored by RT activity in the culture supernatants, by cell viability, and by the percentage of FIV-antigen positive cells at various intervals. As shown in Fig. 2-4, although TM-Ac2 virus could replicate in both cell lines, the kinetics of the mutant virus were different from those of the wild type, such as low levels of virus release (1.5- to 2.0-fold) and delay in the appearance of the peak RT activity (3 to 6 days) (this experiment was repeated at least three times and we obtained the same results) (Fig. 2-4, a and b). The viable cell counts and the percentage of FIV-antigen positive cells in infected MYA-1 cells indicated that the levels of viral cytopathogenicity and propagation

also decreased in the TM-Ac2 virus when compared with the wild type virus (Fig. 2-4, c and d). These experiments suggested that the mutation in the ORF-A gene has an effect on viral replication and propagation in established T cell lines.

To ascertain whether this phenotype of the TM-Ac2 virus is results in a delay in virus release to the culture supernatant, we mesured intracellular RT activity of infected MYA-1 cells. However, no significant defference between the kinetics of intracellular RT and those of supernatant RT was found (Fig. 2-5), and most of intercellular RT were released into the medium after apperance of the peak RT activity in both virus. These observation demonstrated that accumulaton of TM-Ac2 virus particules was not occured in the infected cells.

ORF-A mutant virus displays severe defect in PBLs

The replication potential of TM-Ac2 virus in primary PBLs was also analyzed. The PBLs were isolated from two healthy cats and used after stimulation. Cell-free supernatants from the cells transfected with pTM219 or pTM-Ac2 were adjusted to the same RT activity (10^5 cpm) and infected. The virus production was monitored by RT activity in the culture supernatants. In the first donor, the virus replication was also measured with *gag* protein production in

the infected PBLs by immunoblotting. RT activity of wild type virus-infected PBLs was detected in early day after infection, and the virus could replicate well in both PBLs, as in established cell lines (Fig. 2-6A). In contrast, growth of TM-Ac2 virus was much more slower than that of wild type virus, and levels of RT activity detected in the supernatant were significantly reduced. In the second donor, no significant level of viral replication by the ORF-A mutant was detected during the 32 days of monitoring. This experiment was repeated four times, and each time we found very little virus production after infection with the ORF-A mutant. The appearance of *gag* protein in the ORF-A mutant virus-infected PBLs was also delayed (8 days) (Fig. 2-6B). These data suggested that the function of ORF-A gene may be more critical for productive viral replication in primary PBLs than in established cell lines.

Next, to investigate whether the virus eventually arises from first donor cat PBLs is the same as the input virus, chromosomal DNAs were extracted from infected PBLs at 37 days post-infection, and amplified a central region containing ORF-A sequence by PCR. The locations of the primers were showed in Fig. 2-7A. Since the *Acc*I site was eliminated in pTM-Ac2, the presence or absence of this restriction site is diagnostic for the mutation. Cleaved or uncleaved PCR products were electrophoresed, and transferred

to a membrane. Upon hybridization, the wild type DNA digested with *Acc*I yielded the expected two bands (Fig. 2-7B, lane d), whereas no size reduction of PCR product was observed in TM-Ac2 (Fig. 2-7B, lane e). Furthermore, progeny viruses, produced in the PBLs infected with the mutant virus, behaved exactly like the TM-Ac2 virus (data not shown). These data confirmed that reversion of the ORF-A gene did not occur in PBLs in which ORF-A mutant virus replicated.

To demonstrate that the efficiency of early phase of the TM-Ac2 virus infection, proviral DNAs were isolated from PBLs from the second donor shown in Fig. 2-8 at intervals, amplified with same primers indicated in Fig. 2-7A, and hybridized. As shown in Fig. 2-8, the amounts of PCR products detected in ORF-A mutant virus was nearly same as those detected in wild type virus, suggesting that the ORF-A function is not required for early phase of the virus infection.

DISCUSSION

Mutational analysis of the FIV genome was performed to analyze the effects of mutation in the ORF-A gene on various properties of the virus. The data show that ORF-A mutant virus exhibits a severe defect in the productive replication in primary PBLs.

In the transfection assay using CRFK cells, the phenotypes of *vif* and ORF-A mutant clones were not distinct from those of wild type, although the other mutants displayed virological heterogeneity in either RT production in the culture supernatant or viral protein synthesis in the transfected cells (Fig. 2-1, and 2-2, Table 2-1).

The most interest finding of this work was obtained from investigation of the mutant virus infectivity. The experiment using the cell-free viruses from the transfected cells showed that only ORF-A mutant virus could infect to cultured cells (Fig. 2-3). The ORF-A mutant virus was infectious to two established $CD4^+$ T cell lines, MYA-1 cells and FeL-039 cells, and primary PBLs. However, the ORF-A mutant failed in efficient viral replication and propagation. These properties of the ORF-A mutant virus were showed more drastically in the primary PBLs than in established cells, which were manifested both in the time of initial detection of RT activity and in the *gag* protein

production.

The exact role of the ORF-A gene in the viral life cycle is not clear at the moment. Because particles containing RT activity were produced in the medium of CRFK cells transfected with the ORF-A mutant as well as with the wild type (Fig. 2-1C), we can conclude that the ORF-A gene product is not involved in the expression of genomic RNA, assembly, and release of core particles in the cells. The assay of intracellular RT activity in the infected MYA-1 cells also confirmed that the particles release from these cells was not affected by loss of the ORF-A function. Futhermore, no significant defference between ORF-A mutant virus and wild type virus was observed in the efficiency of early phase of viral infection (Fig. 2-8), suggesting that ORF-A function was not required for viral adaptation, penestration, and reverse transcription.

Recently, analysis of *trans*-activators of FIV LTRs was reported by Sparger et al. (Sparger *et al.*, 1992). They demonstrated very low level *trans*-activation (three- to fivefold) of both pF34 LTR and pPPR LTR by either the pF34 proviral DNA or the pPPR proviral DNA in two different feline non-lymphoid cells (CRFK cells and G355-5 cells), and that co-transfection of both LTRs with pPPR ORF-A gene subgenomic clone resulted in low level (two- to threefold) or no *trans*-activation. In our previous study, using an LTR-

chloramphenicol acetyitransferase (CAT) construct (pSPTM1-CAT), *trans*-activation ability of an infectious clone pTM219 was not demonstrated (Miyazawa *et al.*, 1993). CAT ability directed by an ORF-A deletion mutant of pTM219 was similar to that by pTM219 (Miyazawa *et al.*, 1993). From these results, functional ability of the ORF-A product as a *trans*-activator of the FIV LTRs was not clear. However, potential functional role of the ORF-A gene product in PBLs was not examined in these studies (Miyazawa *et al.*, 1993; Sparger *et al.*, 1992). Considering the behavior of the ORF-A mutant virus in primary PBLs, it is possible that the ORF-A gene product is a *trans*-activator.

Furthermore, Phillips and colleagues (Phillips *et al.*, 1990) reported that the 34TF10 clone (Petaluma isolate), which contains a stop codon in ORF-A, replicated poorly in primary PBLs. In contrast, the PPR clone, which contains an intact ORF-A sequence, grew well in primary PBLs. These findings are consistent with the results obtained here and suggested an important role of the ORF-A gene in primary PBLs.

Infection of primary PBLs, as opposed to established cell lines, is probably closer to what occurs during a natural infection in cats. Since the defect of the ORF-A mutant was observed more severe during infection in primary PBLs, it is likely that the ORF-A mutant virus replicates

poorly in vivo. Therefore, it will be important to study the effects of ORF-A mutant on the progress and pathogenesis of disease in cats.

LEGENDS FOR FIGURES

Fig. 2-1 FIV mutants used in this study. Schematic representation of FIV genome is shown at the top. Various open reading frames are based on the complete nucleotide sequence of FIV (Maki *et al.*, 1992). MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; DU, dUTPase; IN, integrase; SU, surface glycoprotein; TM, transmembran protein. (A) Restriction enzymes used to generate mutants. The location of the mutation induced is indicated (lines). (B) Designation of mutants. Mutated genes are indicated in parentheses.(C) Transient expression of RT activity in CRFK cells 48 h after transfection. 219 (wild type), Bs (*gag* mutant), Av (*pol* mutant), Bt (*pol* mutant), Ac (*vif* mutant), Ac2 (ORF-A mutant), EcV (*env* mutant), Sp (*env* mutant), Nd (*rev* mutant), Cr shows RT activity in pUC19-transfected cells (negative control).

Fig. 2-2 Immunoprecipitation of FIV proteins produced in transfected CRFK cells. Cell were labeled 48 h after transfection. Cell lysates were precipitated and analyzed by SDS-PAGE (7.5 to 15% gradient gel). Lanes: a, 219 (wild type); b, Bs (*gag*); c, Av (*pol*); d, Bt (*pol*); e, Ac (*vif*); f, Ac2 (ORF-A); g, EcV (*env*); h, Sp (*env*); i, Nd (*rev*); j, pUC19 (negative control). PR, *env* precursor protein gp130;

SU, *env* surface glycoprotein gp105; CA, *gag* capsid protein p27; MA, *gag* matrix protein p17. Arrow indicates truncated form of *env*-SU produced by an *env* mutant. Molecular weight markers are shown at the right.

Fig. 2-3 Infection kinetics in MYA-1 cells inoculated with cell-free virus samples derived from transfected CRFK cells. MYA-1 cells (10^6) were infected with 10^5 RT counts of virus, and RT production in culture supernatant was monitored at the indicated intervals. For RT non-producers shown in Fig. 2-1C, equal volumes of culture fluids from transfected cells were used. Values indicated the day after infection. Mutant designations are shown at the left. 219 (wild type), Bs (*gag* mutant), Av (*pol* mutant), Bt (*pol* mutant), Ac (*vif* mutant), Ac2 (ORF-A mutant), EcV (*env* mutant), Sp (*env* mutant), Nd (*rev* mutant).

Fig. 2-4 Growth kinetic comparison of wild type (TM219 virus) and ORF-A mutant viruses (TM-Ac2 virus) in MYA-1 cells (a) and FeL-039 cells (b). Cells (10^6) were infected with 10^5 RT counts of virus, and RT production in culture supernatant was monitored at intervals. Viable cell numbers (c) and percentage of FIV-antigen positive cells (d) in infected MYA-1 cells are also shown. Viable cell numbers were determined by trypan blue staining, and percentage of

FIV-antigen positive cells were measured by indirect immunofluorescence assay (Miyazawa *et al.*, 1989*b*) at intervals. Symbols are shown in a). d.p.i.: days post infection.

Fig. 2-5 Intracellular RT activity in infected cells. (a) Growth kinetic comparison of wild type and ORF-A mutant viruses in MYA-1 cells. (b) Ratio of intracellular RT activity shown in (a) and supernatant RT activity shown in Fig. 2-4, (a). d.p.i.: days postinfection.

Fig. 2-6 Kinetic comparison of wild type and ORF-A mutant viruses in primary PBLs by RT activity production in culture supernatant (A) and *gag* protein production in infected cells (B). (A) PBLs (10^6) were infected with 10^5 RT counts of virus, and RT production in culture supernatant was monitored at intervals. (a) and (b) represent parallel studies using PBLs from two different donors. Symbols are shown in b). d.p.i.: day post infection. (B) *gag* protein production in the first donor. The protein production was monitored by Western (immuno)blotting. Upper and lower arrowheads indicate FIV core protein CA and MA, respectively. Values show the day after infection.

Fig. 2-7 PCR analysis of viral DNAs prepared from PBLs

infected with wild type or ORF-A mutant viruses. (A) Location of the primers PRVIS and PRENA within central region. Location of *Acc*I site in ORF-A sequence and the restriction sites (*Dra*III and *Pst*I) used to cut out the fragment for a ^{32}P-labeled probe (used for panel B) is indicated. (B) Southern blot analysis of the amplified products by PCR. PBLs were infected with the viruses as shown in Fig. 2-5, and DNAs were extracted from TM219 virus- (a and d), TM-Ac2 virus- (b and e), or mock-infected (c and f) PBLs for PCR amplification at the 37 days after infection. The PCR products were uncleaved (a to c) or cleaved with *Acc*I (d to f) and run through a 1.5% agarose gel. The sizes of amplified products predicted from the location of the primers are indicated.

Fig. 2-8 Southern blot analysis of PCR amplified DNAs obtained from PBLs infected with ORF-A mutant virus. PBLs (10^6) were infected with 10^5 RT counts of virus, and extrachromosomal DNA was extracte from PBLs infecte with wild type (219) or ORF-A mutant (Ac2) viruses at the indicated time. Primers and probes used were shown in Fig. 2-7. Values indicated the time after infection. Arrowheads indicated the size of amplified products predicted from the location of primers: 219, 1105 bp; Ac2, 1107 bp. M indicates results from mock-infected cells.

TABLE 2-1

Characterization of FIV mutants

Mutants	Mutanted gene	RT production [a]	CPE [b]	Infectivity [c]
pTM-219	None	+	+	+
pTM-Bs	*gag*	−	−	−
pTM-Av	*pol*	−	−	−
pTM-Bt	*pol*	−	−	−
pTM-Ac	*vif*	+	−	+[d]
pTM-Ac2	ORF-A	+	+	+
pTM-EcV	*env*	+	−	−
pTM-Sp	*env*	+	−	−
pTM-Nd	*rev*	−	−	−

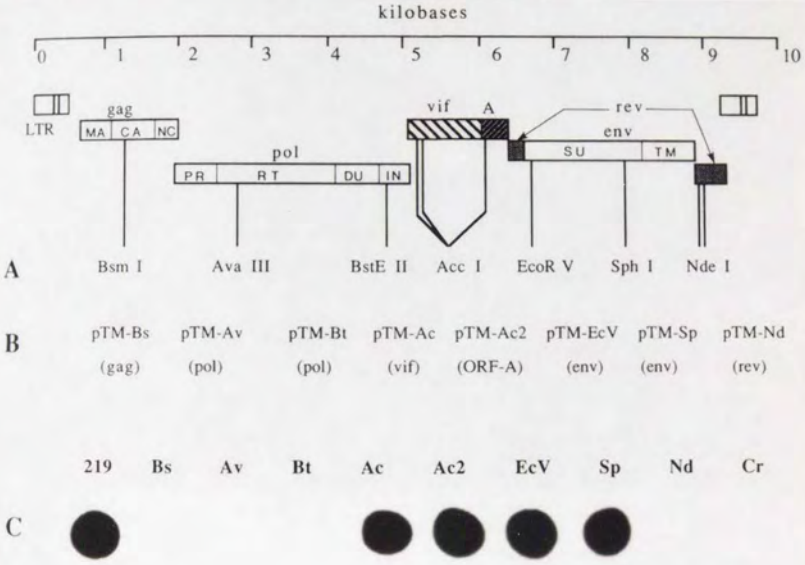
[a] Virus production was assayed by the presence of particle-associated RT activity in the culture supernatants of transfected CRFK cells. Symbols: +, similar level (50 to 100%) of RT production by wild-type; -, no detectable RT.

[b] CPEs (MYA-1 cells) caused by virus were monitored daily by microscopic observation and trypan blue staining. Symbols: +, evidence of CPEs; -, no evidence of CPEs.

[c] MYA-1 cells were infected with cell-free virus stock, and RT production was assayed at intervals for 30 days (Fig. 2-3). Symbols: +, evidence of infectivity; -, no evidence of infectivity.

[d] Infectivity of pTM-Ac was determinated by RT production in the coculture of transfected CRFK cells and MYA-1 cells (CHAPTER 1).

Fig. 2-1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pTM-Bt (pol)
pTM-Ac (vif)
pTM-Ac2 (ORF-A)
pTM-EcV (env)
pTM-Sp (env)
pTM-Nd (rev)
219
Bs
Av
Bt
Ac
Ac2
EcV
Sp
Nd
Cr
C

Fig. 2-2

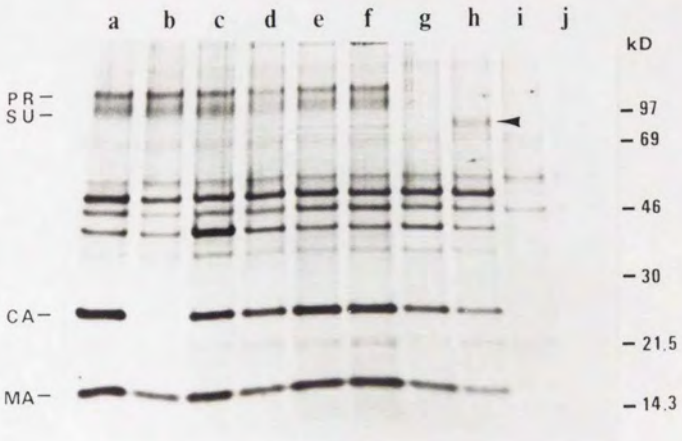


Fig. 2-3

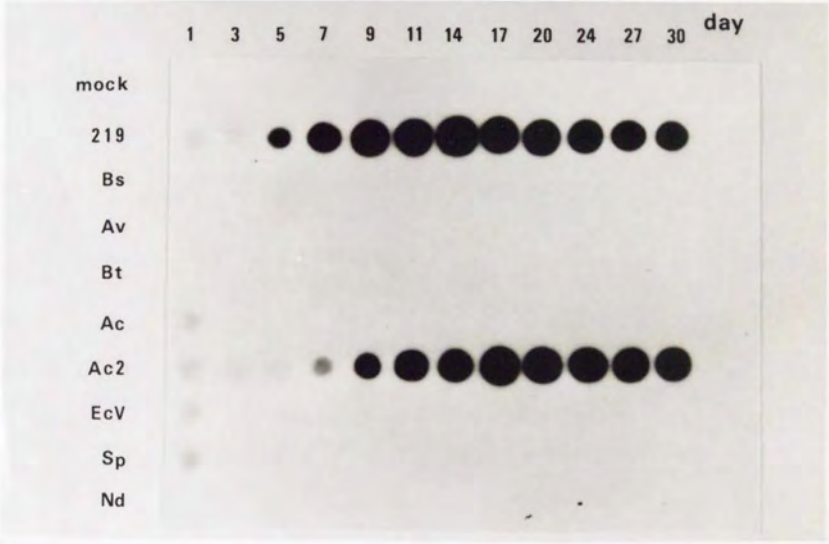


Fig. 2-4

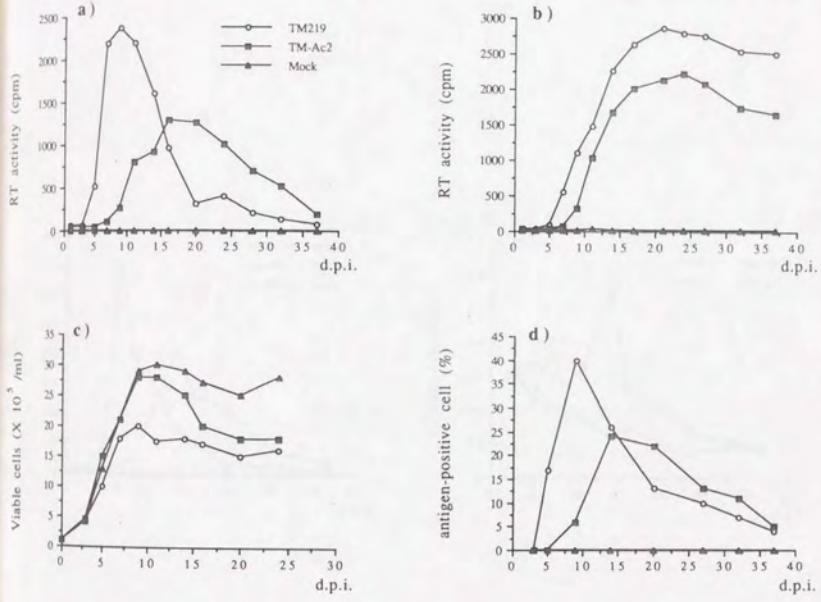

a)
TM219
TM-Ac2
Mock
RT activity (cpm)
d.p.i.
b)
RT activity (cpm)
d.p.i.
c)
Viable cells (X 10^5 /ml)
d.p.i.
d)
antigen-positive cell (%)
d.p.i.

Fig. 2-5

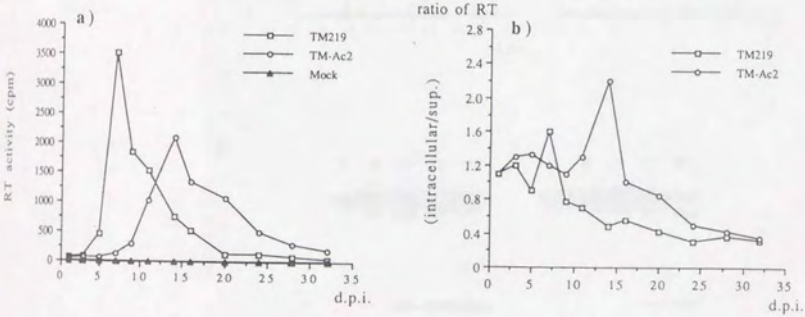

a)
TM219
TM-Ac2
Mock
RT activity (cpm)
4000
3500
3000
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35
d.p.i.
ratio of RT
(intracellular/sup.)
b)
TM219
TM-Ac2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
0 5 10 15 20 25 30 35
d.p.i.

Fig. 2-6

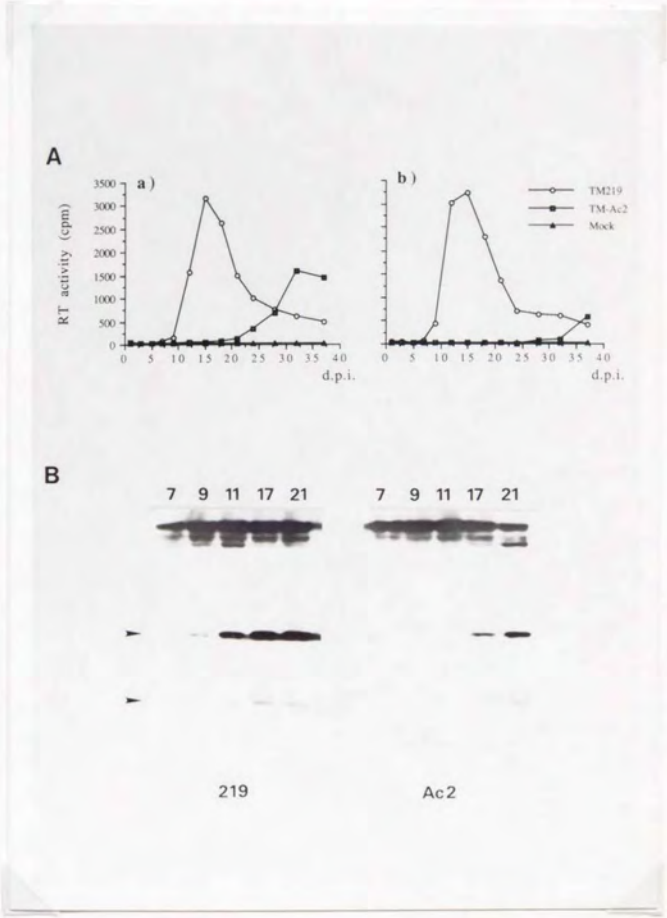

A
a)
b)
RT activity (cpm)
3500
3000
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35 40
d.p.i.
TM219
TM-Ac2
Mock
B
7 9 11 17 21
7 9 11 17 21
219
Ac2

Fig. 2-7

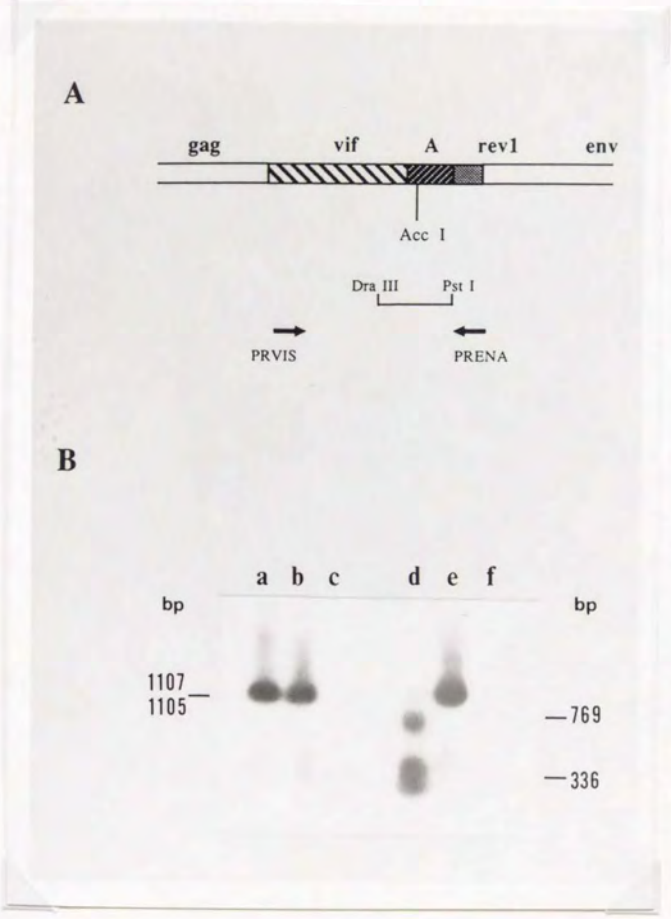
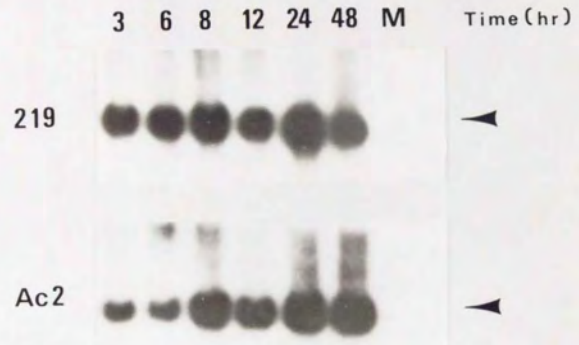

A
gag
vif
A
rev1
env
Acc I
Dra III
Pst I
PRVIS
PRENA
B
a b c d e f
bp
bp
1107
1105
769
336

Fig. 2-8



CHAPTER 3

Feline immunodeficiency virus gene expression: analysis of the RNA splicing pattern and the monocistronic *rev* mRNA

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SUMMARY

The transcription pattern of feline immunodeficiency virus (FIV) genome in a feline $CD4^+$ cell line was examined. In addition to the genomic RNA (9.2 kilobases [kb]), at least five FIV-specific transcripts (5.2, 4.4 [doublet], 1.7, and 1.4 kb) were detected by using subgenomic restriction enzyme fragments of FIV molecular clone or FIV-specific oligonucleotide as probes. Among these transcripts, 9.2, 5.2, and 4.4 (doublet) kb mRNAs were not expressed in the cytoplasm of the cell transfected with a *rev* minus mutant. To determine the location of splice junctions in FIV genome, we used the polymerase chain reaction to amplify and clone cDNAs corresponding to the viral mRNAs from infected cells. The region between *pol* and *env* was found to contain at least two splice donor and three splice acceptor sites. Two splice acceptor sites were detected in the region of 3' of *env*. By hybridization analysis and sequencing of cDNA clones, it is revealed that the medium size mRNAs are derived from a single-splice event, which use different splice acceptor sites, and two smaller transcripts are doubly or triply spliced mRNAs. Our results demonstrate a complex pattern of alternative splicing of FIV mRNAs. Furthermore, we identify monocistronic *rev* mRNA species that employ a unique splice acceptor site.

INTRODUCTION

The lentiviruses including human (HIV), simian (SIV), feline (FIV), and bovine immunodeficiency viruses (BIV), equine infectious anemia virus, visna virus, and caprine arthritis-encephalitis virus belong to the same family of retroviruses (for a review, see Narayan and Clements, 1990). These viruses causes slowly progressive multi-organ diseases in their natural hosts (Ishida *et al.*, 1989; Narayan and Cork, 1985; Pedersen *et al.*, 1987; Yamamoto *et al.*, 1988). The genomes of the lentiviruses have been shown to encode several proteins that affect various aspects of viral expression or infectivity and are thought to play a role to these complex virus-host interactions (Cullen and Greene, 1989; Pavlakis and Felber, 1990). Levels of virus expression are determined to a great extent by the action of regulatory proteins.

The expression patterns of viral mRNAs were well studied in HIV/SIV and visna virus systems (Davis *et al.*, 1987; Schwartz *et al.*, 1990*a*; Viglianti *et al.*, 1990). The primary full-length viral RNA transcript is processed into mRNAs by multiple splicing events and by a mechanism which appears to involve temporal regulation of gene expression (Cullen and Greene, 1990). The small, multiply spliced mRNA species, encoding the viral regulatory proteins Tat and Rev,

are expressed in the early stage of infection. The *tat* gene product stimulates viral gene expression through its action on the long terminal repeat (LTR) and causes the accumulation of high levels of multiply spliced mRNA early in infection. The HIV-1 Rev activates the expression of late genes including the three viral structural genes (*gag*, *pol*, and *env*), and *vif* and *vpr* genes, whose mRNAs contain the *cis*-acting RNA target sequence for Rev designated as Rev responsive element (RRE) (Garrett *et al.*, 1991; Schwartz *et al.*, 1991).

The genetic organization of the FIV appears to be relatively simple compared with that of other lentiviruses in that the gene contains at least three short open reading frames (ORFs), *vif*, ORF-A, and ORF-B, in addition to the structural genes common to all retroviruses (Maki *et al.*, 1992; Olmsted *et al.*, 1989*a*, *b*; Talbott *et al.*, 1989). In CHAPTER 1, we demonstrated that the *vif* gene of FIV is essential for cell-free virus infectivity, like the *vif* genes of various primate lentiviruses (Tomonaga *et al.*, 1992, CHAPTER 1 in this thesis). A short 3' ORF that overlaps the LTR, ORF-B, is at least a part of *rev* gene (Kiyomasu *et al.*, 1991; Phillips *et al.*, 1992). The location of the FIV RRE was identified at the 3' end of the *env* ORF (Phillips *et al.*, 1992), but not at the Su-TM junction, as in other lentiviruses.

Recently, the transcription patterns of several FIV mRNAs were reported (Phillips *et al.*, 1990, 1992; Tomonaga *et al.*, 1992, CHAPTER 1 in this thesis). However, the actual mapping of splice sites was not performed. Further it is not known whether all transcripts are equally translated or whether a single protein is translated from mRNA that may encodes sequences for more than one protein. In this chapter, as one approach towards determining the products of the FIV genome that regulates its expression, we examined the expression pattern of FIV-specific RNAs and splice sites in an FIV-infected $CD4^+$ lymphoblastoid cell line. The results revealed that the FIV exhibited complex splicing pattern, and that monocistronic *rev* mRNA in the strain TM2 employed a unique splice acceptor site, which was not found in American isolates.

Cell culture and virus. A feline $CD4^+$ lymphoblastoid cell line (MYA-1 cells) (Miyazawa *et al.*, 1989*b*) and Crandell feline kidney (CRFK) cells (ATCC CCL94) were maintained as described in CHAPTER 1. MYA-1 cells infected with FIV TM2 strain (Miyazawa *et al.*, 1989*a*) were used for analyses of transcription of the virus. The complete nucleotide sequence of this virus has been determined (Maki *et al.*, 1992) and the GenBank data base accession number are described in CHAPTER 1.

Transfection. Uncleaved plasmid DNA was introduced into CRFK cells by the calcium phosphate coprecipitation method as described in CHAPTER 1.

RT assays. Virion-associated reverse transcriptase (RT) activity was measured as described in CHAPTER 1.

RNA extraction and Northern (RNA) blotting. Total cellular RNA was extracted from MYA-1 cells infected with the FIV TM2 strain by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Cytoplasmic RNA was prepared from transfected CRFK cells as described before (Sambrook *et al.*, 1989). $Poly(A)^+$ RNA was

enriched from the total RNA and from the cytoplasmic RNA by using Oligotex-dT30 (Takara shuzo Co., Ltd., Kyoto, Japan). RNA was electrophoresed through a 1% agarose gel containing 2.2% formaldehyde, and analyzed by Northern (RNA) blot hybridization as previously described (Rabson *et al.*, 1985). Excised DNA fragments prepared from a molecular clone of FIV, pTM219 (Kiyomasu *et al.*, 1991) and FIV TM2 strain-specific oligonucleotides were labeled with ^{32}P by the random primed labeling system (Boehringer Mannhiem Yamanouchi Co., Ltd., Tokyo, Japan.) or end-labeling and then used as probes. The exact positions of the probes were indicated in Table. 3-1

Synthetic oligonucleotide primers. Seven amplification primers were used in this study. The locations of the amplification primers are indicated in Fig. 3-3. The sequences and exact positions of the primers in the TM2 genome are as follows: PRLTS, 5'-GAACTCCTGCAGACCTTGTG-3' (nucleotides [nt] 436 to 455 of strain TM2); PRVIA, 5'-CAGGTAGACTCGATATGTAC-3' (nt 5335 to 5316); PRVIS, 5'-GTACATATCGAGTCTACCTG-3' (nt 5136 to 5335); PROAA, 5'-CTAATAGCTGGTTCTCTATC-3' (nt 6061 to 6042); PRENA, 5'-TGAAGTAATGCCTGGTACCC-3' (nt 6421 to 6402); PRENS, 5'-GGGTACCAGGCATTACTTCA-3' (nt 6402 to 6421); PRREA, 5'-AGCGGTTCTCTAGATTTGCC-3' (nt 9032 to 9013). Each of the sense

and antisense primer pairs was used to amplify cDNAs for detection of the splice junctions in the TM2 genome. The primer pair PRLTS-PRREA was used for amplification of all of the small, multiply spliced mRNAs.

cDNA synthesis and PCR amplification. The polymerase chain reaction (PCR) (Saiki *et al.*, 1988) was used to determine the splice donor and acceptor sites of FIV TM2 strain. Poly(A)$^+$ RNA (0.5 to 1 μg) was reverse transcribed in the presence of the antisense oligonucleotide primer, and the resulting DNA was amplified in the same tube as described in CHAPTER 1

Cloning and sequencing of amplified cDNA. After amplification of the cDNAs by PCR, the products were digested with appropriate restriction enzymes, and cloned into pUC118 or pUC119 cloning vectors. For sequencing, the double-stranded plasmid DNAs were denatured and dissolved in the reaction mixture together with the sequencing primer. The DNA sequences were determined using the Sequenase version 2.0 kit (United States Biochemical Co., Cleveland, Ohio).

Analysis of PCR products. To investigate the structure of the small, multiply spliced mRNAs of FIV, the PCR product

amplified with a primer pair PRLTS-PRREA was analyzed on a 5% polyacrylamide gel. DNA was visualized by UV fluorescence after staining with ethidium bromide. To denature DNA before blotting, the gel was soaked in 0.5M NaOH-0.6M NaCl for 10 min, washed twice with H_2O, and neutralized in 2.0M Tris-HCl(pH 7.0)-0.6M NaCl for 10 min. The DNA was transferred to nylon membrane by electroblotting at 35mA for 16 h in 2X TBE buffer (0.1M Tris-HCl PH 8.3, 0.1M boric acid, 2.0mM EDTA) at 4℃ . After cross-linking of DNA, the membrane was prehybridized for 1 h at 37℃ . Hybridization was performed overnight with ^{32}P-labeled probes. The locations of the hybridization probes are indicated in Fig. 3-8. The exact positions of the probes are: 2A (nt 5191 to 5290); VIA (nt 5316 to 5335); 4 (nt 6264 to 6445); 5 (nt 8912 to 9008).

Expression of viral mRNAs.

In CHAPTER 1, we reported the transcription pattern of FIV by Northern blot analysis (Tomonaga *et al.*, 1992, CHAPTER1 in this thesis). This study revealed transcripts of 9.2, 5.2, and 4.4 kb which presumably represent *gag-pol*, *vif*, and *env* mRNAs, respectively, as well as smaller transcripts (approximately 1.7 kb) which did not hybridize to *vif* and *env* probes (Tomonaga *et al.*, 1992, CHAPTER 1 in this thesis). To characterize these RNAs in more detail, we used a series of probes specific for different regions of the viral genome and TM2-specific oligonucleotide. The locations of each probe within the FIV genome are shown in Fig. 3-1 and Table 3-1.

The LTR probe (probe A in Fig. 3-1) clearly detected two distinct small, multiply spliced mRNAs, 1.7 and 1.4 kb, in addition to the genomic and medium-size mRNAs (Fig. 3-1, lane a, and Table. 3-1). The *gag-pol* probe (probe B in Fig. 3-1) detected only one genomic size mRNA, 9.2 kb species (Fig. 3-1 lane b, and Table. 3-1). The specific probe for *vif* (probe C in Fig. 3-1) hybridized to the *gag-pol* and *vif* mRNAs (Fig. 3-1 lane c, and Table. 3-1). To identify the genetic content of the smaller mRNAs, a few small probes were used. Probe D, which does not contain a consensus

splice acceptor site (Mount, 1982) just upstream from *env* ORF, hybridized to the 9.2, 5.2, 4.4, and 1.7 kb RNA species (Fig. 3-1 lane d, and Table. 3-1). Probe E, which was the same oligonucleotide as primer PRENA located in 5' end of *env* and did not contain the ORF-A sequence, detected 9.2, 5.2, 4.4, 1.7, and 1.4 kb RNA species (Fig. 3-1 lane e, and Table. 3-1). Probe F, which is located just downstream from a consensus splice donor sequence (Mount, 1982) in the 5' end of *env*, detected 9.2, 5.2, and 4.4 kb species. These results suggested that at least a splice acceptor site is present between Probes D and E, and small, multiply spliced mRNAs are spliced to the 3' end of the genome before Probe F. Although the 4.4 kb band was hybridized to these three probes, the band detected by probes E and F was broader than that detected by probe D. From these hybridization patterns, it was predicted that the 4.4 kb band was a doublet, and that the probe D detected only the upper 4.4 kb band, while probes E and F detected both upper and lower 4.4 kb bands. This conclusion was supported by the analysis of splice sites (see below). The 4.4 kb transcripts are singly spliced and only upper 4.4 kb species contains ORF-A sequence. Probe G hybridized to genomic and all medium size species (Fig. 3-1 lane g, and Table. 3-1). The ORF-B specific probe detected all viral specific bands, as did probe A (data not shown). In addition to these clear bands, less distinct bands were

detected around 3.0 kb with LTR (probe A), *env* (probe G) (Fig. 3-1 lanes a, and g), and ORF-B probes (data not shown).

Next, to investigate the role of the FIV Rev protein in expression of these transcripts, we analyzed cytoplasmic poly(A)$^+$ RNA from CRFK cells transfected with the *rev* minus mutant proviral clone, pTM-Nd (Tomonaga *et al.*, 1992, CHAPTER 1 in this thesis), by Northern blot analysis (Fig. 3-2). When the LTR specific probe was used, all viral specific bands were detected in the cells transfected with the wild-type viral clone, pTM219 (Fig. 3-2, lane a). In contrast, the probe detected only small, multiply spliced mRNA species in the cells transfected with pTM-Nd (Fig. 3-2, lane b). Compared with the wild type, the 1.4 kb mRNA in the cell transfected with pTM-Nd was unclear, because the *rev* minus proviral clone was deleted between the two *Nde* I sites, which contained the splice acceptor site for the 1.4 kb transcript (see below). The *env* specific probe hybridized to the genomic and medium-size mRNAs in the cells transfected with pTM219 (Fig. 3-2, lane d). However, the probe failed to detect any specific mRNAs in the cells transfected with pTM-Nd (Fig. 3-2, lane e). These results revealed that the cytoplasmic expressions of the 9.2, 5.2, and 4.4 (doublet) kb mRNAs were dependent on the Rev protein (Table. 3-1).

Analysis of the splice junctions of FIV.

To map splice junctions, partial cDNAs were constructed and amplified by using oligonucleotide primers. The primers used in this study were shown in Fig. 3-3A. After amplification, the products were cloned into pUC118 or pUC119 vector and sequenced to determine the splice donor and acceptor sites in the genome. To determine the splice sites, at least six clones were sequenced on each of the products. The 5' splice donor site at nt 611 (SD1) of the TM2 sequence and the splice acceptor site at nt 5194 (SA1) (48 bases 5' to the first AUG of the *vif* ORF) were previously reported in CHAPTER 1 (Tomonaga *et al.*, 1992). The other splice sites were detected by combinations of each primer pair. One of the products amplified by using primer pair PRLTS-PROAA contained three exons. The second exon of the product employed the splice acceptor site for *vif* ORF (SA1), and terminated at the splice donor site at nt 5262 (SD2), while the third exon employed the splice acceptor site at nt 5928 (SA2) which was located close to the end of the *vif* ORF, 69 base upstream of ORF-A initiation codon.

Another product employed the splice donor (SD1) and acceptor (SA2), and consisted of only two exons. The splice acceptor site just 5' of the *env* ORF at nt 6264 (SA3) was detected using primer pair PRLTS-PRENA and is predicted to

be used to generate the *env* mRNA. The splice donor site which located in the 5' end of *env* sequence and utilized for multiply spiced mRNA species was identified at nt 6515 (SD3). This splice donor site was joined to the splice acceptor site either at nt 8959 (SA4) or 9015 (SA5) (Fig. 3-4). 71 % of the clones obtained from the amplification by using primer pair PRENS-PRREA employed the splice donor site at nt 6515 (SD3) and the acceptor site at nt 8959 (SA4). The splice acceptor site at nt 9015 (SA5) was located downstream of the conserved Arg-Lys rich domain of the *rev* sequence of FIV (Fig. 3-4), suggesting that the *rev* mRNA of FIV TM2 strain employed the splice acceptor site at nt 8959 (SA4) but not at nt 9015 (SA5). We previously reported that the splice acceptor sites in the 3' portion of *env* were predicted to be at nt 8900 and 8959 (Maki *et al.*, 1992), however, we did not detect use of the splice acceptor site at nt 8900 in this study.

Fig. 3-3B shows the locations of splice sites on the FIV genome and the putative structure of each mRNA transcript which was deduced from hybridization to different probes and sequencing of partial cDNA clones. The upper 4.4 kb mRNA was recognized by probes D (Fig. 3-1), suggesting that the transcript may employ the splice acceptor site at nt 5928 (SA2) and encode ORF-A and/or *env* products. The 1.7 kb mRNAs probably represent doubly or triply spliced mRNAs

and were believed to be the ORF-A/*rev* messenger. Transcripts similar to these two 1.7 kb mRNAs were also reported by Phillips and colleagues in the Petaluma isolate (clone 34TF10) (Phillips *et al.*, 1992). The 1.4 kb mRNA may consist of the presumed glycoprotein leader sequence in 5' of the *env* ORF and 3' sequences of the genome. Recently, it has been reported that these two regions were essential for a *rev* gene activity (Kiyomasu *et al.*, 1991; Phillips *et al.*, 1992). Thus, it is predicted that the transcript represents the monocistronic messenger for the FIV *rev* gene.

To verify that the 1.4 kb transcript functions as a *rev* messenger, we constructed FIV Rev expression plasmid, pTM-Rev2.1, in which the *Sac*I -*Xba*I fragment of plasmid pTM219, containing an intact provirus inserted into plasmid pUC19, was replased by the *Sac*I -*Xba*I fragment of a cDNA clone encoding the 1.4 Kb transcript (Fig. 3-5A). The *rev* mutant (pTM-Nd) was transfected into CRFK cells with or without pTM-Rev2.1 and evaluated for transient expression of virion-associated RT. Although RT production was not observed in CRFK cells transfected with pTM-Nd, it was observed in CRFK cells cotransfected with pTM-Nd and pTM-Rev2.1, at wild type levels (pTM219) (Fig. 3-5B). This result showed that pTM-Rev2.1 complements the *rev* minus mutant, and hence that the 1.4 kb transcript functions as a messenger for the FIV *rev* gene. The sequence of

monocistronic *rev* cDNA was shown in Fig. 3-6. The location of the FIV *rev* first coding exon was overlapping and in the same reading frame as *env*, and its deduced amino acid sequence was similar to those of other lentivirus Rev proteins (Maki *et al.*, 1992). The first coding exons of visna virus and BIV *rev* also occupy similar positions at the 5' end of and in the same reading frame as *env* (Davis *et al.*, 1987; Oberste *et al.*, 1991). The boundaries of first intron FIV *rev* were identical to those of the single intron in the *env* transcript, since they shared the same splice donor and acceptor for this region (Fig. 3-6).

Sequence analysis of the splice sites in FIV TM2 (Fig. 3-7A) revealed that nearly all splice sites were well conserved in the different FIV sequences. However, the sequence of the splice acceptor for *env/rev* mRNAs in FIV TM2 (SA3) was not conserved in American isolates (clones 34TF10, and PPR) (Phillips *et al.*, 1992; Talbott *et al.*, 1989), and we could not find analogous splice sites in their sequences (Fig. 3-7B).

From the results of the cDNA sequencing, the locations of the exons in the FIV genome were predicted. To identify the genomic regions within the FIV cDNAs, the cDNAs amplified with a primer pair PRLTS-PRREA were transferred to nylon membranes and hybridized to different probes. The location of each probe and the results of hybridization are

shown in Fig. 3-8. All bands visualized by ethidium bromide staining hybridized to the probe which located in exon 1 (data not shown). Compared with probe VIA, probe 2A which encompassed non-coding exon 2A, hybridized to many bands, strongly indicating that many multiply spliced mRNAs contained non-coding exon 2A. Probe VIA, which located between exons 2A and 3, hybridized to only one band at above 1.3 kb, indicating that only one exon 2-containing mRNA was generated. Probe 4 was located in the overlap region of exons 3 and 4. Probe 5 did not contain exon 6 sequences. Thus, the bands detected with both probes 4 and 5 at between 0.64 kb and 0.47 kb may form mRNAs expressing ORF-A and/or *rev* proteins. The product at below 0.47 kb was detected with probe 4, while probe 5 failed to do it. This observation confirmed that the transcript using SD3 and SA5 was certainly generated, as shown in Fig. 3-4. The product at 0.34 kb was only hybridized to probe 2A, suggesting the existance of at least an unidentified small exon in the genome. The smallest band was detected with probes 2A and 5 but not with probes VIA and 4. Based on its size and hybridization properties the transcript consists of the exons 1, 2A, and 5.

DISCUSSION

By Northern blot analysis, cDNA synthesis and DNA sequencing, our results demonstrate that FIV TM2 strain in feline $CD4^+$ lymphoid cell line has a complex pattern of viral gene expression employing alternative splicing strategies similar to those observed in other lentivirus-infected cells (Davis *et al.*, 1987; Noiman *et al.*, 1990; Oberste *et al.*, 1991; Schwartz *et al.*, 1990*a*; Viglianti *et al.*, 1990).

In addition to the 9.2 kb genomic-length RNA, five other distinct viral RNA species were detected. The cytoplasmic expression of these transcripts was examined with the *rev* minus proviral clone (Fig. 3-2, and Table. 3-1). The *rev* mutant expressed only small, multiply spliced mRNAs but not unspliced and singly spliced mRNAs, 9.2, 5.2, and 4.4 (doublet) kb species in the cytoplasm. This result clearly indicated that the cytoplasmic expressions of 9.2, 5.2, and 4.4 (doublet) kb mRNAs were Rev-dependent and these transcripts expressed in late stages of the FIV replication cycle in infected cells.

All subgenomic RNAs contain a short 5' leader spliced to different regions of the genome 3' of the *pol* gene. It was found that the splice donor site at nt 611 (SD1) was utilized for all subgenomic RNA species. The two mRNAs of

4.4 kb have different genetic structures; the larger band contains sequences from ORF-A, whereas the smaller species contains the *env* ORF. Two splice acceptors are present in this region (at nt 5928 and 6264) and are probably utilized in these mRNAs. Either or both of these transcripts could function as the *env* mRNA. However the larger transcript contains the entire ORF-A and thus may function to produce a protein product from this region.

The 1.7 and 1.4 kb transcripts are at least doubly spliced, containing sequences from the 5' end of the genome spliced to sequences located between *pol* and *env* which are then spliced to sequences at 3' of the *env* ORF. The 1.7 kb transcripts contain sequences from the whole of ORF-A spliced to sequences in ORF-B. These transcripts may have a bicistronic coding capacity and function as ORF-A and *rev* mRNAs. The finding of these 1.7 kb transcripts was supported by the recent report of Phillips *et al* (1992). The 1.4 kb transcript was the monocistronic *rev* mRNA and the first coding exon was overlapping and in the same reading frame as *env* ORF (Fig. 3-6). It is a unique finding that the splice acceptor site for monocistronic *rev* mRNA (SA3) in the strain TM2 is not conserved in other strains (Fig. 3-7B), in contrast to other splice sites which are well conserved. In fact, only bicistronic mRNAs (ORF-A and *rev*) have been identified in American isolates. Schwartz *et al* (1990*a*)

reported that HIV *tat* mRNAs produced very low levels of Rev compared to *rev*-only mRNAs. Although detail ORF-A function is unclear now (Tomonaga *et al.*, 1993*b*, CHAPTER 2 in this thesis), assessment of the relative translation efficiency of the bicistronic and monocistronic mRNAs will be important for a full understanding of the regulation of virus gene expression.

In this study, two splice acceptor sites (SA4 and SA5) were detected in the 3' region of *env*, whereas Phillips *et al.* found only one splice acceptor site (SA4 equivalent) here. The transcripts employing SA5 were minor species and could not form *rev* mRNA. However it is possible that this splice acceptor site is employed for the last exon of the ORF-A-only mRNA or an other mRNA which encodes a novel protein. Our Northern blot analysis using LTR and gene-specific probes revealed that the approximately 3.0 kb mRNAs hybridize with LTR (Fig. 3-1; probe A), env (Fig. 3-1; probe G), and ORF-B probes, indicating that these mRNAs contain the exon which located in the *env* ORF, and as such are similar to *tev* in HIV-1 transcripts (Benko *et al.*, 1990; Salfeld *et al.*, 1990). Further study is needed to determine the identity of these RNA species.

A non-coding exon 2A is located in the overlap region of the *pol* and *vif* genes (Fig. 3-8). The exon is conserved in other strain of FIV (Phillips *et al.*, 1992) as a leader

sequence of transcripts. Many cDNAs hybridized to the probe which encompassed exon 2A, suggesting that non-coding exon 2A was contained in many multiply spliced mRNA species. Although it is not clear whether the length of leader sequence has any effect on the function of the transcripts, it is noteworthy that the mRNAs containing exon sequences similar to 2A in FIV are produced by other lentiviruses such as HIV-1, SIV$_{MAC}$, and visna virus (Davis and Clements, 1989; Sodroski *et al.*, 1985; Viglianti *et al.*, 1990). An mRNA containing exon 2 was detected by hybridization (Fig. 3-8). This mRNA contains the entire *vif* sequence and may form FIV the analogue of the HIV-1 mRNA expressing Vif protein, which is doubly spliced (Arya and Gallo, 1986). From the results of experiment in figure 3-8, it is predicted that many transcripts might be generated in the infected cells more than we could detect.

Our study and previous reports of others demonstrate that lentiviruses including FIV share a pattern of mRNA expression with complex alternative splicing, suggesting that the expression of lentiviruses may be controlled at many different levels. Therefore, further extensive studies will be important for our understanding of the viral life cycle and the significance of viral gene regulation for pathogenesis.

LEGENDS FOR FIGURES

Fig. 3-1 Northern blot analysis of poly(A)$^+$ RNA from MYA-1 cells infected with FIV TM2 strain. FIV genome organization is shown at top. The solid bars indicate the locations of the hybridization probes. The size of each species of mRNA is shown on the left. Lanes; a, probe A; b, probe B; c, probe C; d, probe D; e, probe E; f, probe F; g, probe G; h, poly(A)$^+$ RNA prepared from uninfected MYA-1 cells and processed exactly like RNAs from infected cells. RNA in lane h was hybridized to probe A. The exact positions of the probes are indicated in Table. 3-1.

Fig. 3-2 Cytoplasmic expression of FIV RNA in the transfected cells. CRFK cells were transfected with pTM219 (lanes a and d), pTM-Nd (lanes b and e), or pUC19 (lanes c and f). Cytoplasmic RNA was isolated 72 h after transfection, and Northern blot analysis was performed with the LTR (probe A in Fig. 3-1) or *env* (probe G in Fig. 3-1) probes.

Fig. 3-3 Detection of the transcription patterns of FIV by PCR. Schematic representation of FIV genome is shown at the top. (A) Arrows indicate oligonucleotide primers used for cDNA synthesis. The exact positions of these

oligonucleotide are indicated in Materials and Methods. (B) The locations of the splice donor (SD) and acceptor (SA) sites and structures of the viral mRNA transcripts deduced from hybridization results to different probes and sequence of partial cDNA clones. The corresponding nucleotide number of each splice site is indicated in the text.

Fig. 3-4 Splice junction of the 3' of FIV genome. Schematic representation of partial FIV genome indicates the position of splice donor (SD3) and acceptor (SA4, and SA5) sites. The solid bars indicated the exons within the transcripts that are employed splice sites SD3 and SA4 (a), and SD3 and SA5 (b). Black box in the ORF-B region indicates the sequence position which encodes Arg-Lys rich domain of Rev.

Fig. 3-5 Analysis of the FIV Rev function of the 1.4 kb transcript. (A) Construction of the FIV *rev* expression plasmid, pTM-Rev2.1. LTR, long terminal repeat. D1, D3, A3, and A4 indicate the splice donor or acceptor sites SD1, SD3, SA3, and SA4, respectively. (B) RT production in CRFK cells 48 h after transfection. Cells were transfected with pTM219 (wild type), pTM-Nd (*rev* mutant), or mock (negative control), or cotransfected with pTM-Nd and pTM-Rev2.1 (FIV *rev* expression plasmid).

Fig. 3-6 Nucleotide sequence and amino acid sequence of monocistronic *rev* cDNA of FIV TM2 strain. The splice junctions were determined by cDNA sequencing. The positions of the splice donor and acceptor sites are indicated. The exact positions of the splice sits are as follow: SD1, nt 611; SD3, nt 6515; SA3, nt 6264; SA4, nt 8959. Boxed ATG indicates translation initiation codon of the RNA.

Fig. 3-7 Nucleotide sequences of the splice sites of mRNA in FIV TM2. (A) FIV TM2 splice donor and splice acceptor sites. The nucleotide sequence of various cDNA clones isolated by PCR amplification was determined. The nucleotide positions indicated refer to the positions of splice donor and splice acceptor sites. The splice donor and acceptor consensus sequences were obtained from a report of Mount (1982). (B) Comparison of nucleotide sequences of splice acceptor site for *rev* mRNA in TM2 strain with different isolates (clone 34TF10 and PPR). The nucleotide changes from the TM2 sequence were indicated by asterisk. Bars represent gaps introduced to optimize the alignment. TAG in the box is indicated the stop codons for ORF-A (or ORF-2). ATG in the box is indicated the initiation codons for *rev* coding exon 1.

Fig. 3-8 Southern blot analysis of cDNAs amplified with primer pair PRLTS-PRREA by PCR. The positions of the amplification primers (arrows), predicted exons (identified by numbers), and the positions of hybridization probes (solid bars) are indicated at the top. The positions of the exons were deduced from sequencing of the cDNA clones. For the exact positions of the probes, see Materials and Methods. The molecular size are indicated on the right. EtBr, Ethidium bromide.

Table 3-1

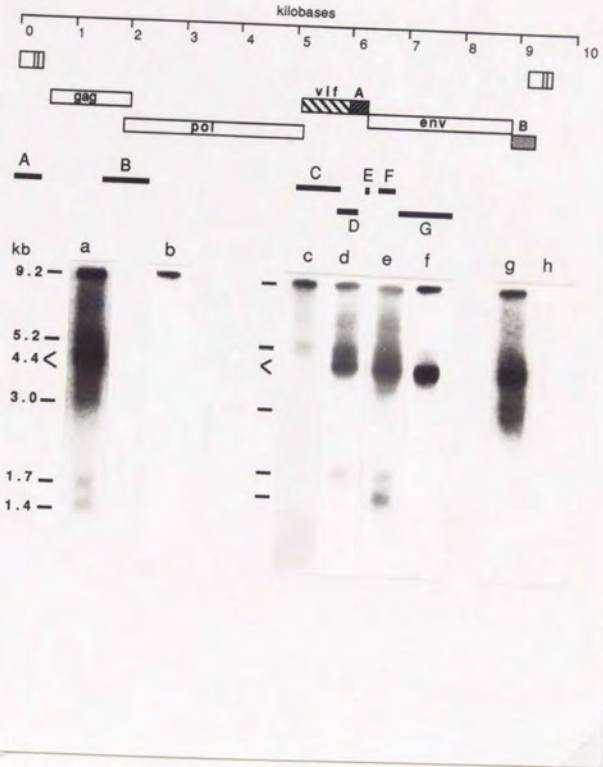
Summary of Hybridization Results [a]

probe	position in TM2 strain	mRNA present (kb)					
		Rev-dependence[b]				Rev-independence[b]	
		9.2	5.2	4.4 (upper)	4.4 (lower)	1.7	1.4
A	16-515	+	+	+	+	+	+
B	1751-2503	+					
C	5171-5936	+	+				
D	5936-6086	+	+	+		+	
E	6402-6421	+	+	+	+	+	+
F	6694-7110	+	+	+	+		
G	7110-7957	+	+	+	+		

[a] Results from Northern blot analysis of a single lot RNA (Fig. 3-1).

[b] Transcripts which cytoplasmic expression is dependent on the Rev protein or independent on the Rev protein (Fig. 3-2).

Fig. 3-1


kilobases
0 1 2 3 4 5 6 7 8 9 10
gag
vif A
pol
env
B
A
B
C
E F
D
G
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b
c d e f
g h
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5.2
4.4
3.0
1.7
1.4

Fig. 3-2

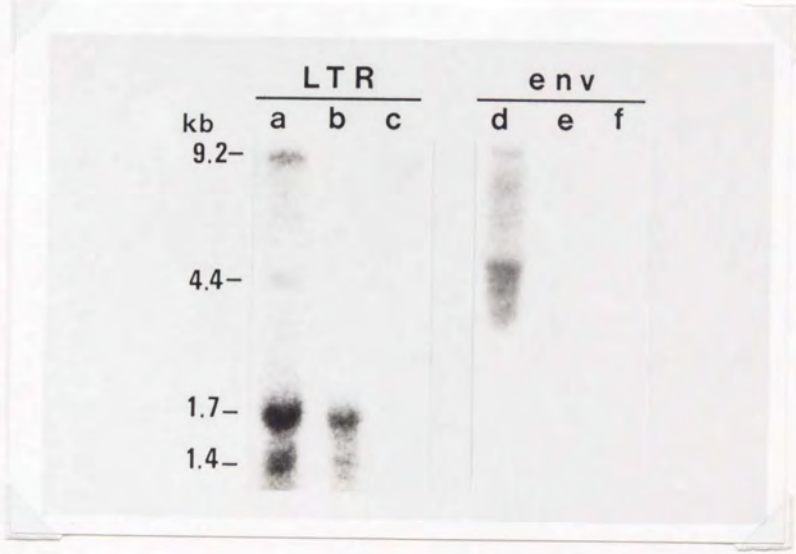
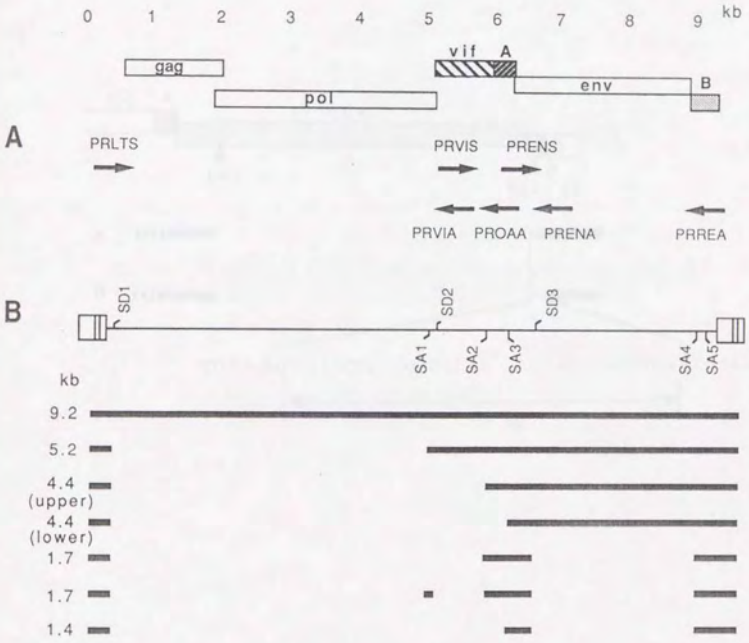


Fig. 3-3


0
1
2
3
4
5
6
7
8
9
kb
gag
pol
vif
A
env
B
A
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PRVIS
PRENS
PRVIA
PROAA
PRENA
PRREA
B
SD1
SD2
SD3
SA1
SA2
SA3
SA4
SA5
kb
9.2
5.2
4.4
(upper)
4.4
(lower)
1.7
1.7
1.4

Fig. 3-4

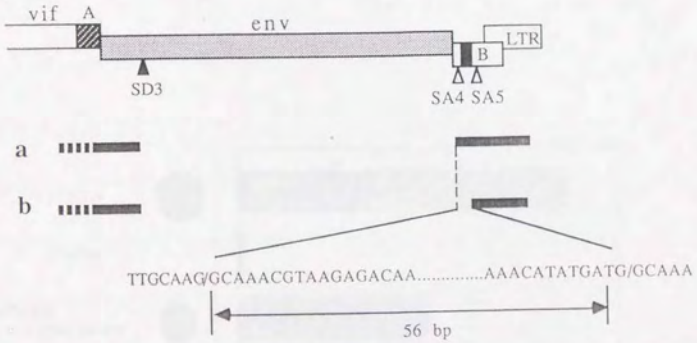
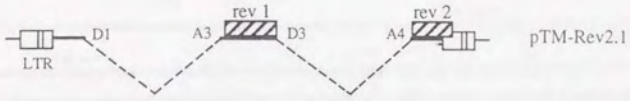

vif
A
env
LTR
B
SD3
SA4 SA5
a
b
TTGCAAG/GCAAACGTAAGAGACAA.....AAACATATGATG/GCAAA
56 bp

Fig. 3-5

A


rev 1
rev 2
D1
A3
D3
A4
LTR
pTM-Rev2.1

B

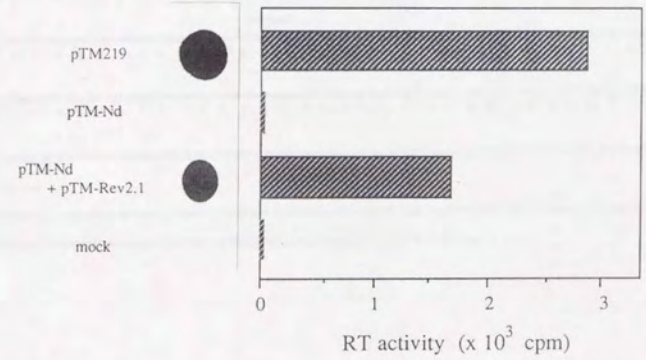
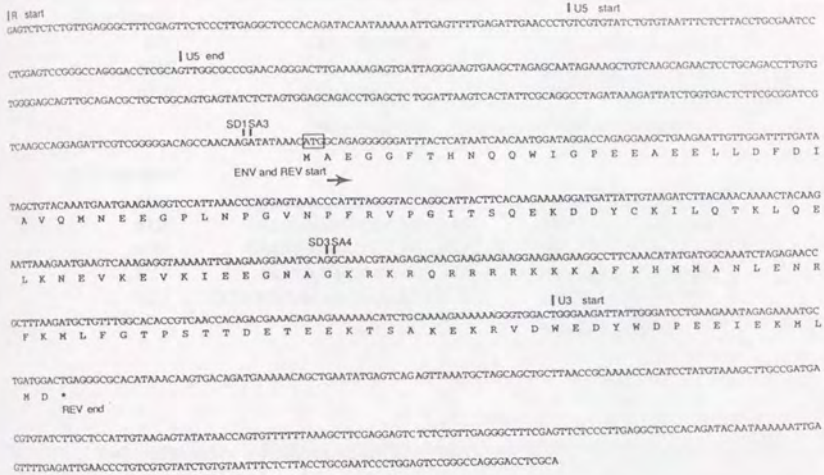

pTM219
pTM-Nd
pTM-Nd
+ pTM-Rev2.1
mock
0
1
2
3
RT activity (x 10^3 cpm)

Fig. 3-6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U5 start
GAGTCTCTCTGTTGAGGGCTTTCGAGTTCTCCCTTGAGGCTCCCACAGATACAATAAAAA ATTGAGTTTTGAGATTGAACCCTGTCGTGTATCTGTGTAATTTCTCTTACCTGCGAATCC
U5 end
CTGGAGTCCGGGCCAGGGACCTCGCAGTTGGCGCCCGAACAGGGACTTGAAAAAGAGTGATTAGGGAAGTGAAGCTAGAGCAATAGAAAGCTGTCAAGCAGAACTCCTGCAGACCTTGTG
TGGGAGCAGTTGCAGACGCTGCTGGCAGTGAGTATCTCTAGTGGAGCAGACCTGAGCTC TGGATTAAGTCACTATTCGCAGGCCTAGATAAAGATTATCTGGTGACTCTTCGCGGATCG
SD1
SA3
TCAAGCCAGGAGATTCGTCGGGGGACAGCCAACAAGATATAAAC ATG GCAGAGGGGGGATTTACTCATAATCAACAATGGATAGGACCAGAGGAAGCTGAAGAATTGTTGGATTTTGATA
M A E G G F T H N Q Q W I G P E E A E E L L D F D I
ENV and REV start
TAGCTGTACAAATGAATGAAGAAGGTCCATTAAACCCAGGAGTAAACCCATTTAGGGTAC CAGGCATTACTTCACAAGAAAAGGATGATTATTGTAAGATCTTACAAACAAAACTACAAG
A V Q M N E E G P L N P G V N P F R V P G I T S Q E K D D Y C K I L Q T K L Q E
SD3
SA4
AATTAAAGAATGAAGTCAAAGAGGTAAAAATTGAAGAAGGAAATGCAGGCAAACGTAAGAGACAACGAAGAAGAAGGAAGAAGAAGGCCTTCAAACATATGATGGCAAATCTAGAGAACC
L K N E V K E V K I E E G N A G K R K R Q R R R R K K K A F K H M M A N L E N R
U3 start
GCTTTAAGATGCTGTTTGGCACACCGTCAACCACAGACGAAACAGAAGAAAAAACATCTG CAAAAGAAAAAAGGGTGGACTGGGAAGATTATTGGGATCCTGAAGAAATAGAGAAAATGC
F K M L F G T P S T T D E T E E K T S A K E K R V D W E D Y W D P E E I E K M L
TGATGGACTGAGGGCGCACATAAACAAGTGACAGATGAAAAACAGCTGAATATGAGTCAG AGTTAAATGCTAGCAGCTGCTTAACCGCAAAACCACATCCTATGTAAAGCTTGCCGATGA
M D *
REV end
CGTGTATCTTGCTCCATTGTAAGAGTATATAACCAGTGTTTTTTAAAGCTTCGAGGAGTC TCTCTGTTGAGGGCTTTCGAGTTCTCCCTTGAGGCTCCCACAGATACAATAAAAAATTGA
GTTTTGAGATTGAACCCTGTCGTGTATCTGTGTAATTTCTCTTACCTGCGAATCCCTGGAGTCCGGGCCAGGGACCTCGCA

Fig. 3-7

A

NUCLEOTIDE POSITION	SEQUENCE	COMMENTS
Splice donor		
611	AAG / GUAGGA	major subgenomic (SD1)
5262	CAG / GUAAGU	non-coding exon (SD2)
6515	CAG / GUAAGU	ORF-A, rev coding exon 1 (SD3)
	$^{C}_{A}$AG / GU$^{A}_{G}$AGU	consensus sequence
Splice acceptor		
5194	UUUCUUGUACCUAG / GAU	vif, non-coding exon (SA1)
5928	GUUUCUCCUUUCAG / AAC	ORF-A, rev coding exon 1 (SA2)
6264	UAUUUCAUCUGCAG / AUA	env, rev coding exon 1 (SA3)
8959	UCUGUAUUGCAAG / GCA	rev coding exon 2 (SA4)
9015	CCUUCAAACAUAUGAUG / GCA	ORF-A exon ? (SA5)
	($^{U}_{C}$)nN$^{C}_{U}$AG / G	consensus sequence

B

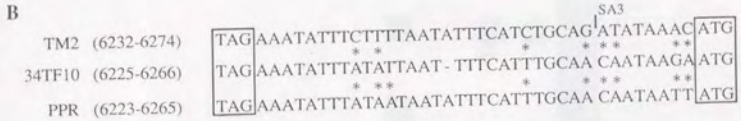
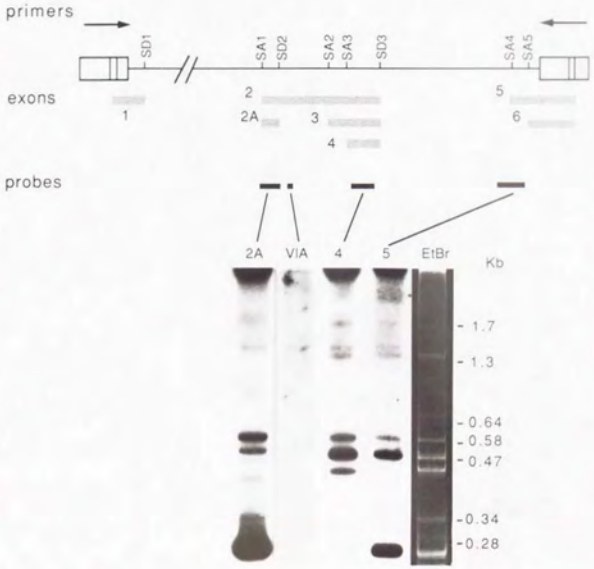


Fig. 3-8


primers
SD1
SA1
SD2
SA2
SA3
SD3
SA4
SA5
exons
1
2
2A
3
4
5
6
probes
2A
VIA
4
5
EtBr
Kb
1.7
1.3
0.64
0.58
0.47
0.34
0.28

CHAPTER 4

Comparison of the Rev transactivation of feline immunodeficiency virus in feline and non-feline cell lines

Journal of Veterinary Medical Science (1993). In press.

SUMMARY

The Rev protein of feline immunodeficiency virus (FIV) differentially transactivates the expression of viral structural proteins by allowing the accumulation of unspliced and singly spliced viral mRNA in cytoplasm via the Rev response element (RRE) in the *env* region. To investigate the role of *rev* gene of FIV for the virus life cycle and cell tropism, we constructed the Rev expression plasmids, and functional activity of the Rev was assayed by using chloramphenicol acetyltransferase (CAT) assay system in feline and non-feline cell lines. Although the FIV Rev protein showed high transactivity to result in enhanced CAT production in a feline cell line, the productions of the CAT in non-feline cell lines were significantly lower than that in a feline cell line. These results display that specific cellular factor(s) present in feline cell line is required for the FIV Rev full-action and also suggest that the Rev action plays one of the important roles in determining the FIV cell tropism.

INTRODUCTION

The lentiviruses have been isolated from various species of primates, felids and ungulates (Narayan and Clements, 1990). The viruses have tropisms specific for cell type and species, which are caused by several limiting steps precluding the normal viral replication cycle. The first block of the virus infection exists in the step of the viral entry that is dependent on the presence of a number of specific factors in the target cells, such as human CD4 molecule which is bound by human immunodeficiency virus type 1 (HIV-1) envelope (Laudau *et al.*, 1988). Another block of the viral replication exists in the step of viral gene expression. This step also crucially determines the virus tropism, because the virus promoter is activated by several transactivation factors including cellular factors.

Feline immunodeficiency virus (FIV) has a highly species-specific tropism, and replication of the virus does not complete in non-feline cell lines. Recently, we have demonstrated that virion production from FIV provirus-transfected primate cell lines was much less efficiently when compared with that from the provirus transfected feline cell line (Miyazawa *et al.*, 1992). This phenomenon is at least in part due to a decreased activity of the FIV long terminal repeat (LTR), which is essential for the virus gene

expression. Additionally, the FIV gene expression is regulated by a regulatory gene, *rev*, which is encoded within the FIV genome (Kiyomasu *et al.*, 1991; Phillips *et al.*,1992). The Rev acts via a *cis*-acting RNA sequence designated as Rev response element (RRE) located in the 3' end of the *env* gene, and is required for the expression of unspliced and singly spliced mRNAs that encode the viral structural proteins (Kiyomasu *et al.*, 1991; Phillips *et al.*, 1992).

Recently, it was reported that cellular factor(s) is required for HIV-1 Rev action (Trono and Baltimore, 1990; Vaishnav *et al.*, 1991; Winslow and Trono, 1993), and HIV-1 RRE contributes to cell type specific viral tropism (Dayton *et al.*, 1993). These findings suggested that interaction between the Rev protein and RRE is important not only for the viral replication but also determination of the virus tropism. However, to date, the role of the FIV Rev is not identified, except that for the virus gene expression. In this chapter, as one approach for determining the role of *rev* gene for the cell tropism of FIV, we analyze the *rev* gene activity in several cell lines including non-feline cell lines.

MATERIALS AND METHODS

Cell cultures. The HeLa (human epithelioid carcinoma cells), SW480 (human colon carcinoma cells) (Adachi *et al.*, 1986), COS (monkey kidney cells transformed by simian virus 40) (Gluzman, 1981), Vero (monkey kidney cells), and CRFK cells (feline kidney cells) (Crandell *et al.*, 1973) used in present study were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotics.

DNA transfection. For transfection of plasmid, cells grown 90% confluence in cultures were used. Uncleaved plasmid DNAs were transfected into cells by the calcium phosphate coprecipitation method as discribed in CHAPTER 1.

DNA constructs. To construct FIV Rev expression vectors, we used two cDNA clones of FIV strain TM2 which were isoleted from FIV-infected lymphoid cell lines (Tomonaga *et al.*, 1993*c*) and described in CHAPTER 3. For efficient expression of FIV Rev in feline and non-feline cell lines, pRVSV vector which contains the Rous sarcoma virus (RSV) LTR and the simian 40 (SV40) small t intron and polyadeneylation signal (Sakai *et al.*, 1990) was selected. Two Rev expression effector clones which encode intact ORF-A and/or *rev* genes (Fig. 4-1A) were constructed by insertion of the necessary

DNA fragment into the pRVSV. Thus, a plasmid pF·A/rev contains entire ORF-A and *rev* coding sequences, and expresses both ORF-A and Rev proteins. A plasmid pF·rev contains only *rev* coding sequences (Fig. 4-1B). Another plasmid pF·revR contains the antisense orientation of the *rev* gene and used as negative control effector (Fig. 4-1B). Parallel transfection with the constitutive chloramphenicol acetyltransferase (CAT) expression vector, pRVSV-CAT (previously referred to as pRSpCATΔ RΔ A) (Sakai *et al.*, 1990), served as positive control for transfection efficiency. For the reporter plasmid, pRSpCAT-RREf (Kiyomasu *et al.*, 1991) which contained RRE fragment of FIV TM2 strain in downstream of the CAT gene was used.

CAT assay. For the CAT assay, cell monolayers in each well were harvested by scraping at 48 h after transfection. After being washed once in cold PBS, the cells were lysed by freezing and thawing four times in 200 μl 0.25 M Tris-HCl pH 7.8. Cell debris was pelleted by centrifugation, and assayed for CAT activity (Gorman *et al.*, 1982) by the thin-layer chromatography (TLC) method (Sakai *et al.*, 1990). Briefly, 20 μl of cell extract was mixed with 170 μl of CAT reaction mixture containing [^{14}C]chloramphenicol and incubated 37 ℃ for 30 to 60 min. After incubation, 1 ml of ethyl acetate was added to the mixture and vortex, and then

centrifuged. The top layer was removed and dry the ethyl acetate in a Speedvac evaportor. The dry up sample was resuspended in 15 μ l of ethyl acetate and spotted on a TLC plate. The TLC plate was allowed the chromatography about 1 h. The TLC plate was dryed and visualized by autoradiography or counting by the imaze analyzer.

RESULTS AND DISCUSSION

The constructs of *rev* encoding cDNAs of FIV strain TM2 and Rev expression vectors used in this chapter were shown in Fig. 4-1. To evaluate the *rev* gene activity of the FIV, we used CAT assay system (Sakai *et al.*, 1990). Reporter plasmids carrying CAT gene and RRE produce CAT at high levels only when the Rev protein is present (Kiyomasu *et al.*, 1991).

First, to determine the ability of the two Rev expression plasmids to enhance CAT production, 2 μg of the pRSpCAT-RREf was cotransfected with 5 μg of either the pF·A/rev or the pF·rev in CRFK cells. For positive control, 2 μg of pRVSV-CAT was also cotransfected with 5 μg of pRVSV. As shown in Fig. 4-2, both Rev expression plasmids showed high activity to result in enhanced CAT production in CRFK cells. The level of the activation by these expression plasmids was almost the same as that by pRVSV-CAT. On the other hand, this enhancement was not observed in the negative effector, pF·revR. These results demonstrated that the FIV Rev expression plasmids derived from the two different cDNAs clones efficiently expressed the Rev protein and the expression of the ORF-A product did not affect the function of the Rev protein in CRFK cells.

Next, to investigate whether the these function of the

Rev protein is equally demonstrated in non-feline cell lines including Vero, COS, HeLa, and SW480 cells, the assay was also carried out. CAT activity was determined as described above. The CAT productions in these cell lines were compared with that in CRFK cells, and the ability of the Rev to activate pRSpCAT-RREf in these cell lines was showed as the CAT activity relative to that of pRVSV-CAT. As shown in Fig. 4-3, although the pRVSV-CAT efficiently expressed the CAT production in non-feline cell lines, the levels of CAT activities by the Rev expression plasmids in these cell lines were significantly low when compared with that in CRFK cells. Especially, the relative CAT activities in HeLa and SW480 cells were 6- to 30-fold less than that in CRFK cells. This experiment was repeated at least three times, and we always obtained the low level of CAT activities in the non-feline cell.

The most important purpose of this work is to investigate whether the FIV Rev can act in non-feline cell line or not. The results of the experiment in Fig. 4-3 clearly demonstrated that functional abilities of the FIV Rev protein in the non-feline cell lines were much less than that in feline cell line. From this cell type specific ability of the Rev protein, it was suggested that the specific cellular factor(s) is required for the Rev full-action, as observed in a HIV-1 Rev (Trono and Baltimore,

1990; Vaishnav *et al.*, 1991; Winslow and Trono, 1993). From this point of view, it is likely that *rev* gene also plays one of the key roles for determination of the tropism of the virus. Therefore, identification of such cellular factor(s) which interacts with the FIV Rev protein is important to understand the mechanisms of the viral replication and tropism.

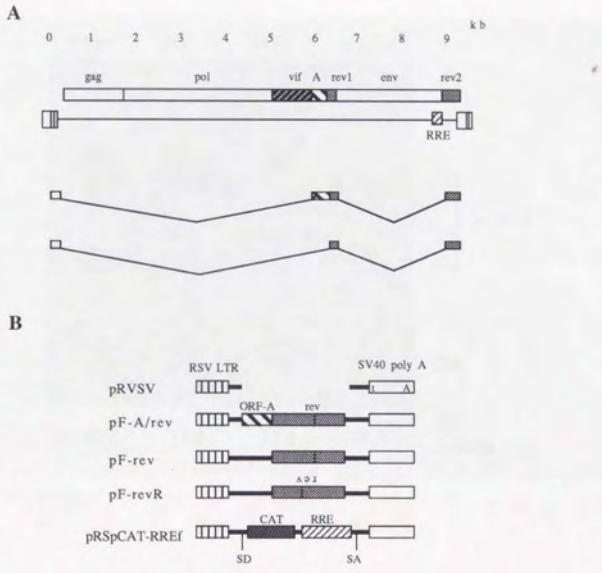
LEGENDS FOR FIGURES

Fig. 4-1 Map of the FIV cDNAs and the expression plasmids used in this study. (A) Schematic representation of FIV genome is shown at the top. The splicing patterns of the two different cDNAs encoding *rev* gene are also shown. The location of the RRE in the *env* gene are showed by box.
(B) Schematic representation of the expression plasmid (pRVSV) and three effector plasmids and a reporter plasmid (pRSpCAT-RREf). t and A indicate the SV40 small t intron and polyadenylation signal, respectively. SD and SA indicate splice donor and splice acceptor site, respectively.

Fig. 4-2 CAT activities directed by the Rev expression plasmids in CRFK cells. pRSpCAT-RREf (2 μ g) was cotransfected with pF·A/rev, pF·rev, or pF·revR (5 μ g), and CAT activity in the cell lysates (60-min reaction) 48 h later was determined. CAT activity in the cell cotransfected by pRVSV·CAT (2 μ g) and pRVSV (5 μ g) was also shown for the positive control. A representative result from thin-layer chromatography, separating [^{14}C]chloramphenicol (CM) from its acetylated forms (Ac-CM), is shown. The percent conversion of chloramphenicol to its acetylated forms is indicated on the below. A/rev: pF·A/rev, rev: pF·rev, revR: pF·revR, RVSV, pRVSV-CAT.

Fig. 4-3 Relative CAT activities in feline and non-feline cell lines. The abilities of the pF·A/rev, pF·rev, and pF·revR to activate pRSpCAT-RREf in the cell lines were showed as the CAT activity relative to that of pRVSV-CAT. Symbols of the bars are indicated on the right.

Fig. 4-1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rev2
RRE
B
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SV40 poly A
pRVSV
ORF-A
rev
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pF-revR
CAT
RRE
pRSpCAT-RREf
SD
SA

Fig. 4-2

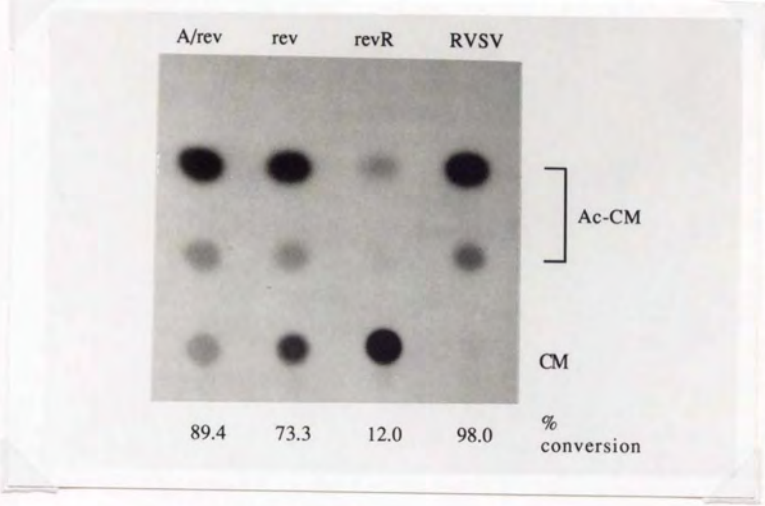
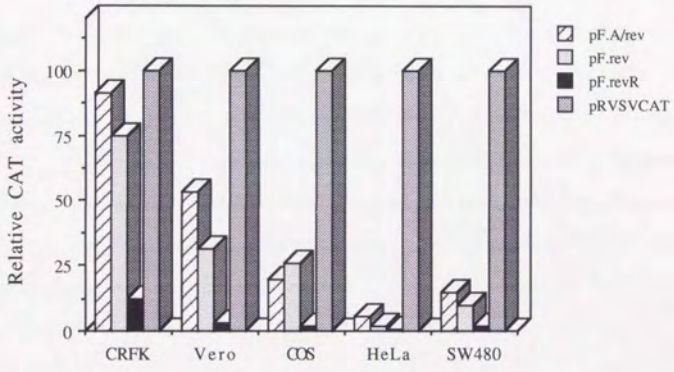


Fig. 4-3


Relative CAT activity
100
75
50
25
0
CRFK
Vero
COS
HeLa
SW480
pF.A/rev
pF.rev
pF.revR
pRVSVCAT

CONCLUSIONS

In this thesis, the author described molecular and biological analysis of FIV.

In CHAPTER 1, function and expression of an auxiliary gene which located in the central region of FIV genome, putative *vif*, was analyzed. Northern (RNA) blot analysis of mRNAs from an FIV (strain TM2)-infected cell lines revealed that the putative-*vif*-specific mRNA was actually transcribed as a 5.2 kb species. To examine the function of the putative *vif* gene, the author constructed mutant derived from an infectious molecular clone of FIV by recombinant DNA techniques. Although cell-free putative *vif* mutant virus prepared from the transfected cells could not infect feline $CD4^+$ cell lines and primary feline PBLs, the infectivity of the mutant virus was demonstrated in a coculture of the transfected cells and feline $CD4^+$ cells. These properties of the putative *vif* mutant virus of FIV were very similar to those of the *vif* mutant viruses of HIV and SIV. These results demonstrated that the FIV contains the *vif* gene, which is structurally and functionally similar to that of the primate lentiviruses, and also suggested that the *vif* gene plays an important role for the lentivirus life cycles because the *vif* gene appears to be conserved in FIV and HIV.

In CHAPTER 2, the author analyzed the function of the ORF-A gene of FIV which was also located in the central region of the genome. The author constructed frameshift

mutants corresponding to all of the identified ORFs of FIV, including the ORF-A gene. In the transient transfection analysis, no significant difference between the phenotype of ORF-A mutant clone and that of wild-type clone was demonstrated, although the other mutant clones displayed virological heterogeneity in either RT production or the viral specific protein synthesis. The cell-free ORF-A mutant virus from the transfected cells, however, reduced efficient viral replication and propagation in established T-cell lines. Interestingly, the defects of the mutant virus were observed to be more severe in primary PBLs than in established cell lines. From these results, it was suggested that the ORF-A gene involved in the efficient viral replication and propagation, but not in the expression of genomic RNA or in assembly and release of core particles in cells. Furthermore, since the defects of the ORF-A mutant virus is more critical during infection in primary PBLs, it was also suggested that the ORF-A gene plays an important role in vivo life cycle of the virus.

The location and sequence of the ORF-A gene resemble to those of *tat* gene of other lentiviruses, and mRNAs which encode the ORF-A sequences are expressed early phase of viral infection (CHAPTER 3). From these observations, it had been predicted that the ORF-A gene product is a *trans*-activator gene of the FIV LTR. Several studies, however,

could not demonstrate the *trans*-activity of the ORF-A gene in non-lymphoid cell lines. To date, the *trans*-activator role of the ORF-A gene product in T-cell lines and primary PBLs was not yet examined. Considering the behavior of ORF-A mutant virus in T-cell lines and primary PBLs, it is possible that the ORF-A gene product plays a *trans*-activator in these cells. Further studies will be required to clarify the exact role of the ORF-A gene.

To understand the gene expression and its regulation of the FIV in more detail, the transcription pattern of the FIV strain TM2 in a CD4-positive cell line was examined in CHAPTER 3. In addition to the genomic RNA, at least five FIV-specific transcripts were detected by the Northern (RNA) blot analysis. Among these transcripts, the genomic size and medium size mRNAs were not expressed in the cytoplasm of cells transfected with *rev* minus mutant clone. This observation demonstrated that cytoplasmic expression of the genomic size and medium size mRNAs were regulated by the *rev* gene product. Furthermore, to determine the splice junction of the FIV, the author used the reverse transcriptase-PCR method to amplify and clone cDNAs. From this analysis, the author found several spliced donor and acceptor sites and identified transcriptional patterns of the mRNAs which were derived from multiple splice events. Of these multiply spliced mRNAs, 1.4 kb transcript was monocistronic *rev* mRNA

which employ a unique splice acceptor site. Interestingly, although nearly all of the splice sites were well conserved in the different FIV strain sequences, the unique spliced acceptor site was not conserved in American isolates. This finding suggested that the gene expression and its regulation of the FIV strain TM2 is likely to be different from other FIV strains. Moreover, the author detected several transcripts by the Southern blotting, demonstrating that the FIV has a complex pattern of viral gene expression employing alternative splicing manners. In this experiment, the author could clone several cDNA which seems to be valuable for the analysis of the gene regulation.

In CHAPTER 4, to examine whether the *rev* gene involved in the cell- or species-specific tropism or not, the Rev *trans*-activity was analyzed in feline and non-feline non-lymphoid cell lines by gene transfection. The FIV Rev expression plasmids were constructed from the *rev* cDNA clones obtained in CHAPTER 3. To assess the Rev *trans*-activity, the author used chloramphenicol acetyltransferase (CAT) assay systems. Although the FIV Rev protein showed high *trans*-activity resulting in an enhanced CAT production in a feline cell lines, the production in non-feline cell lines were significantly reduced compared with that in a feline cell line. Especially, the CAT activity in human HeLa and SW480 cells were 6- to 30-fold less than that in a

feline cell. These results suggested that specific cellular factor(s) present in the feline cell lines required for the FIV Rev full-function and also suggested that the Rev plays an important role for determination of the virus tropism.

In conclusion, the author performed extensive analyses of the function and expression of the FIV genes by using molecular biological techniques. From this study, it was demonstrated that the expressions and replications of the FIV are highly regulated by the viral encoding genes or alternative splicing strategies.

A non-structural gene, *vif*, is expressed in late phase of the virus infection and is essential for cell-free virus infectivity. The function of the *vif* gene is predicted to be conserved in other lentiviruses. Another non-structural gene, ORF-A, is expressed in early phase of the virus infection. Although the ORF-A gene is not required for the virus infectivity, the gene facilitates efficient viral replication. A regulatory gene of the FIV, *rev*, regulates the expression of the late gene including *vif* gene, and involves in the cell- or species-specific tropism. In this study, the author could find various important information, and this findings will be invaluable for the understanding of pathogenesis of FIV in vivo.

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CURRICULUM VITAE

Keizo Tomonaga was born on November 20, 1964 in Fukuoka, Japan. In March 1990, he graduated from Faculty of Agriculture, Kagoshima University. Passed the Japanese National Veterinary Board Examination in March 1990 and received the title of Doctor of Veterinary Medicine.

In April 1990, he moved to Institute for Virus Research, Kyoto University and began Research student program for immunodeficiency virus.

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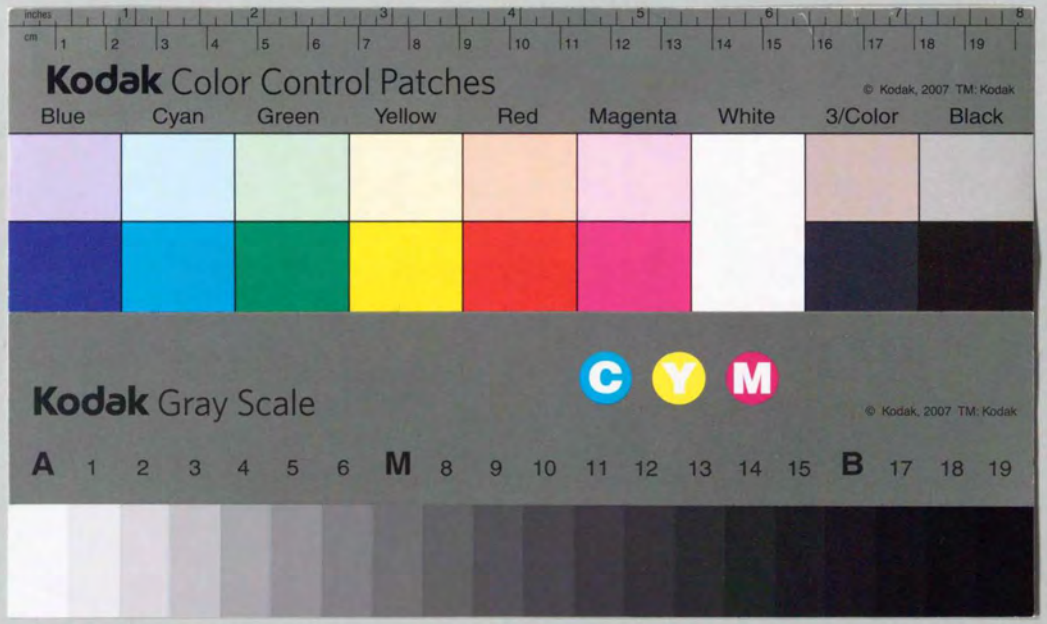
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