Molecular Biological Studies on Feline Foamy Virus

ネコフォーミーウイルスの分子生物学的研究

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CONTENTS

GENERAL INTRODUCTION

INTRODUCTION

Foamy viruses (FVs; also known as spumaviruses) are classified into the genus *Spumaviridae* of the family *Retrovirinae.* The FVs are widespread and have been readily isolated from a variety of mammalian species such as cattle (Johnson *et al.*, 1983), horses (Tobaly-Tapiero *et al.*, 2000), cats (Kasza *et al.*, 1969), humans (Achong *et al.*, 1971) as well as non-human primates such as gorillas (Bieniasz *et al.*, 1995), chimpanzees (Herchenroder *et al.*, 1994), and baboons (Broussard *et al.*, 1997), and African green monkeys (Neumann-Haefelin *et al.*, 1993). The phylogenetic relationship of FVs to the other retroviral genera is shown in Figure 1. FVs are also found in sea lions (Kennedy-Stoskopf *et al.*, 1986) and hamsters (Hruska and Takemoto, 1975) but no viral isolates are currently available. It is a hallmark of FVs that *in vitro* infection of fibroblasts with all of the known FVs induces formation of syncytial giant cells. In some cases, cytopathic effects have foamy appearance (Fig. 2), hence the origin of the name "foamy" virus (Kasza *et al.*, 1969; Mergia *et al.*, 1996; Mikovits *et al.*, 1996). However, *in vivo* infection is usually inapparent and the association of FVs with virally-related diseases remains obscure. Feline foamy virus (FFV) was first isolated in 1969 from a cat with nasopharyngeal calcinoma (Kasza *et al.*, 1969). Several additional strains of FFV and FFV-related viruses have been isolated since the original report (Riggs *et al.*, 1969; Mochizuki and Konishi, 1979; Ikeda *et al.*, 1997; Miyazawa *et al.*, 1998).

PREVALENCE OF FFV

FFV infection is persistent and infected cats have a sustained antibody response. Virus can be isolated from most tissues of infected cats (Hooks and Gibbs, 1975). FFV DNA is present in leucocytes, however, coculture is required for virus isolation (Jarrett *et al.*,1974; von Laer *et al.*, 1996). Large quantities of infectious viruses are excreted in the saliva of infected cats (Shroyer and Shalaby, 1978). The major mode of FFV transmission is currently unknown, but is likely to be through either biting (Pedersen, 1986) or licking/respiratory transfer following intimate social contact (Johnson *et al.*, 1988).

Two different serotypes of FFV isolates have been characterized by viral neutralization. Despite the finding that little or no neutralizing cross-reactivity exists between two serotypes (Flower *et al.*, 1985), infection with a virus of one serotype protects against superinfection by the second serotype. On the other hand, 11 different serotypes of simian foamy virus (SFV) have been characterized (Hooks and Gibbs, 1975; McClure *et al.*, 1994) and an individual primate co-infected with different SFV serotypes is often recognized (Hooks *et al.*, 1972). Reasons for FFV and SFV to have different *in vivo* consequences after superinfection with heterologous serotypes are unknown.

GENETIC STRUCTURE OF FFV

The genome size of FFV is approximately 11,700 nucleotides, as determined by nucleotide sequencing and Northern blot hybridization (Winkler *et al.*, 1997). The FFV genome is larger than that of lentiviruses and several hundred nucleotides shorter than the genome of oncogenic viruses. The genome of FFV encodes three structural genes, group specific antigen (*gag*), polymerase (*pol*) and envelope (*env*), and two auxiliary genes, *bel*-1 and *bel*-2, which are located between the 5'- and 3'-long terminal repeat (LTR) (Fig. 3). Both genomic organization and gene order are conserved among FVs and mechanisms of reverse transcription exhibited by FVs are similar to those of other retroviruses. The initial nucleic acid packaged into virions is RNA (Baldwin *et al.*, 1998), indicating that reverse transcriptase (RT) is activated very early in viral assembly/budding. However, recent experiments with the inhibitor of reverse transcription, 3'-azido-3'-deoxythymidine, indicate that many of the infectious viral particles contain DNA rather than RNA molecules (Moebes *et al.*, 1997; Yu *et al.*, 1999). FV-infected cells contain hundreds to thousands of unintegrated cytoplasmic DNA per cell (Moebes, *et al.*, 1997; Schweizer *et al.*, 1989). Therefore, the FVs life cycle is similar to that of hepadnaviruses, which have DNA genomes encoding for RT (Yu *et al.*, 1996-a).

The gag gene;

The uncleaved Gag precursor protein seems to play a functional role

in FV assembly and maturation (Coffin 1990). Mature Gag proteins serve additional, but related, functions during virus maturation processes such as folding and controlling of morphogenesis. The FFV *gag* open reading frame (ORF) encodes a 55 kDa precursor. Three functional domains, matrix (MA), capsid (CA) and nucleocapsid (NC), are conserved in the *gag* sequence (Flügel, 1991). The proteolytic cleavage of the Gag precursor results in 52 and 48 kDa proteins (Fig. 4). Recently, field-isolates of FFV containing uncleaved mature Gag protein were found, but they replicated poorly in cell cultures (Chapter 1). The proteolytic cleavage between MA and CA or between CA and NC generally plays an important role in the early stages of viral replication for other retroviruses (Pettit *et al.*, 1991). However, the significance of the cleavage step in FFV replication is still unknown.

Most of the unique biology of FV replication may depend on typical Gag function and some of motifs in the Gag domain that have been characterized. The Gly-X-Trp-Gly-X-X-X-Arg-X-X-X-X-X-X-X-Leu-Gln-Asp motifs are conserved in the amino-terminus of MA domain (Winkler *et al.*, 1997). This peptide motif is similar to the dominant morphogenetic signal of type D Mason-Pfizer monkey retrovirus, which is responsible for the cytoplasmatic pre-assembly site of this type of retrovirus (Rhee and Hunter, 1990). Major homology region (MHR) is conserved in the CA domain, but the function is unknown (Berkowitz *et al.*, 1996). Three glycine-arginine-rich motifs (GR box I-III) are tandemly repeated in the NC domain (Schliephake *et al.*, 1994). These motifs are completely absent in the other retroviruses.

The cysteine repeated motifs (Cys-His motifs) are found in the region corresponding to the GR box I-III (Flügel *et al.*, 1987; Herchenroder *et al.*, 1994; Mergia *et al.*, 1991; Renne *et al.*, 1992). The GR box I-III shows strongly basic sequence and is responsible for nuclear localization (GR box II) and /or binding with equal affinity to RNA and DNA (GR box I) (Yu *et al.*, 1996-b). Bodem *et al.* (1998) reported that FFV Gag proteins are exclusively localized in the cytoplasm close to perinuclear regions. However, Gag of SFV is transported to nucleus in infected cells (Schliephake and Rethwilm, 1994). Unique features of FFV Gag might be due to the GR box. Interestingly, hepatitis B virus (HBV) and retroposons possess a strongly basic region in their core protein sequences. Therefore, the Gag protein of FFV may be analogous to the core protein of HBV and retrotransposons (Hatton *et al.*, 1992; Nassal *et al.*, 1992).

The pol gene;

Conventional retroviruses use either a minus ribosomal frame-shift or a suppression of stop codons to generate the Gag-Pro-Pol precursor polyprotein. The polyprotein is subsequently processed by the viral protease to produce Gag, Pro, and Pol cleavage products (Coffin 1990). Unlike that of other retroviruses, the *pro-pol* gene product of FV is translated from a subgenomic message, which lacks the Gag domain (Bodem *et al.*, 1996; Enssle *et al.*, 1996; Jordan *et al.*, 1996; Löchelt and Flügel, 1996; Yu *et al.*, 1996-a). As a consequence, FV Pol precursor contains the protease (Pro), RT, ribonuclease H (RNase H), and integrase (IN) domains (Fig. 4). Two mature

proteins, Pro-RT-RNase H and IN, are found in the infected cells.

The FV *pol* sequence, especially the RT domain, shows the highest identity among FV genes and most of the evolutionary trees among retroviruses are based on this domain. The protein motifs and known features in all retroviral *pol* gene products are conserved in the FV *pol* gene, except for the unusual catalytic center of Pro. Although the canonical catalytic center of the retroviral Pro is identified to be Asp-Ser-Gly-Ala, the glycine residue is replaced by a glutamine residue for FV and results in an Asp-Ser-Gln-Ala motif (Winkler *et al.*, 1997). It is of interest to note that the RT of FV, unlike most other retroviral polymerases, prefers Mn^{2+} to Mg^{2+} as the divalent cation in the DNA polymerase reaction (Liu *et al.*, 1977; Parks *et al.*, 1971). The structural basis for this preference is not known.

The env gene;

Env precursor of about 120 kDa is cleaved into the surface (SU) and transmembrane (TM) proteins. The FFV SU protein is 37 to 40% identical and the FFV TM protein is 49 to 53% identical to the corresponding domains of other FVs. The high degree of homology of FV Env proteins even across host species and family borders may reflect a common feature of FV replication that sets them apart from other complex retroviruses. The unique characteristic is the highly charged carboxy-terminal sequence, Arg-Lys-Lys-Asp-Gln-Stop, which is considered to be a retrieval signal for retrograde transport into the endoplasmatic reticulum (ER). The ER retrieval signal is characterized by two Lys residues located 3 and 4 or 3 and

5 residues directly upstream of the carboxyl terminus of the protein (Jackson *et al.*, 1990; Sohn *et al.*, 1996). The conservation of this signal among FV points to a possible role in preventing syncytium formation in infected cells, evasion of immune surveillance, or perhaps both functions. The ER retrieval signal may also have functions in intracellular FV particle assembly, where preformed particles bud into intracellular membrane compartments (Goepfert *et al.*, 1995; Goepfert *et al.*, 1997).

A typical TATA box motif is present at the 3' end of FFV *env* sequences, about 240 bp upstream of *bel*-1. The nucleotide sequence motif 5'-GAGCTTC-3', which resembles the FV transcriptional start site consensus sequence 5'-GAGCTC-3', is about 30 bp downstream from the TATA box. This nucleotide sequence motif represents the transcriptional start from an internal promoter that has been detected in different primate FV (Campbell *et al.*, 1994; Löchelt *et al.*, 1993-b; Mergia *et al.*, 1994).

The auxiliary genes, bel-1 and bel-2;

Two auxiliary genes, *bel*-1 and *bel*-2, are located in the region between *env* and the 3' LTR of the FV genome. The *bel*-1 gene product is a strong transcriptional trans-activator (Tas) that acts on its LTR and internal promoter (IP) (Meiering *et al.*, 2001). Interestingly, the Tas protein binds to distinct sequences in the LTR promoter and the IP. The Tas transactivates the two promoters by different mechanisms since there is no sequence homology between the LTR promoter and IP (Erlwein and Rethwilm, 1993; Kang *et al.*, 1998; Lee *et al.*, 1993). The amino acid sequence homology of

Tas is low among FVs. All of the known motifs conserved in the HFV-Tas, a long helical region predicted to form a promoter-targeting domain and nuclear localization signal, are absent in FFV-Tas. The Tas of FFV is specific for the homologous LTR and IP and does not cross react with those of other species origin (Yang *et al.*, 1997).

Bet is a major protein synthesized by infected cells both *in vitro* and *in vivo*, but its function is unknown. While Bet is conserved among the family of FVs, it neither share significant sequence identity with any known proteins nor contains known peptide motifs (Löchelt *et al.*, 1991). Since Bel-2 is completely contained within the Bet sequences, antisera to Bet detect the Bel-2 protein that is expressed in FFV-infected cells (Löchelt *et al.*, 1991; Muranyi and Flügel, 1991). The ORF of *bet* starts with the methionine residue that also initiates *bel*-1 transcription. Subsequently *bet* mRNA is spliced from the *bel*-1 ORF into the *bel*-2 ORF in such a way that a protein of 480 amino acids is generated. Thus the name *bet* is derived from *bel*-1 plus *bel*-2 (Mergia *et al.*, 1990).

The third reading frame corresponding to the *bel*-3 gene of HFV as well as SFV-1, SFV-3 and BFV is absent in FFV (Winkler *et al.*, 1997).

The LTR region;

FVs have large LTR region compared with other retroviruses. The full-length FFV LTR is about 1,355 bp. While the R and U5 regions have a high degree of homology among FVs, the main difference is in the U3 regions, which have diverged completely in both size and nucleotide sequence (Maurer

et al., 1988; Kupiec *et al.*, 1991; Mergia *et al.*, 1990). This emphasizes that the variable transcription-regulation mechanisms are responsible for the highly divergent nature of U3. Most notable in this region for FFV is a lack of the AP-1 cellular transcription factor's binding site, the presence of which is symbolic for the U3 of SFV and HFV. Although other *cis*-acting elements such as Bel-1 responsible element (BRE) and putative TATA box are not fully identified in FFV, it is apparent that DNA sequence of FFV BRE differs from other FVs (Zhou and Luciw, 1996). The splice donor site at nucleotide position 51 is also remarkable, since it is the shortest RNA leader of all known retroviruses (Muranyi and Flügel, 1991).

In the HFV and SFV-1 LTR promoter, negative regulatory elements are present in U5 domain (Mergia *et al.*, 1992; Erlwein *et al.*, 1993). It is unknown whether the inhibitory effect is due to a silencer that resides in the U5 region and is recognized by a DNA binding protein or whether the inhibitory effect is related to the secondary structure of HFV RNA transcripts that contain this element (Muranyi and Flügel, 1991; Löchelt *et al.*, 1994; Yu *et al.*, 1996-a; Bodem *et al.*, 1996).

REPLICATION PATHWAY OF FFV

Key points discussed in this chapter are summarized in Figure 5. The replication pathway of FFV differs from that of the conventional retroviruses in the following ways. (i) Mature virions do not contain matrix,

capsid or nucleocapsid proteins. They are composed of two large Gag proteins, which differ at the carboxyl terminus from conventional retroviruses by the absence of 4 kDa. (ii) Reverse transcription is a late event in viral morphogenesis, and the infectious particles probably have DNA genomes. (iii) Viral budding requires both Gag and Env proteins. A majority of the virus buds through the ER. (iv) Most of the FFV particles are intracellular and probably accounts for the large amount of unintegrated DNA seen in infected cells. (v) Persistently infected cells contain large amounts of unintegrated DNA. This could occur through an intracellular recycling pathway, although there is no firm evidence supporting for such a pathway, either *in vitro* or *in vivo*. In each of these steps, FFV has characteristics similar to those of hepadnaviruses. In the case of HBV, reverse transcription is complete prior to budding, the viral core is not cleaved into smaller polypeptides, viral budding requires only core and S proteins, and a recycling pathway allows the progression of a closed and circular virus replication cycle (Linial *et al.*, 1999).

CHARACTERISTICS OF FFV VECTOR

FV have several characteristics that make them ideal for use as gene transfer vectors. They can infect several mammalian species, but have not been associated with clinically apparent diseases. Furthermore, FV can be propagated in a variety of cell types from different species both *in vivo* and *in*

vitro cell cultures. Recently, numerous investigation from several groups have demonstrated the utility of FV as vectors. HFV DNA, like HIV DNA, can enter the nucleus of G_1/S-phase-arrested cells (Saib *et al.*, 1997). In comparative gene transfer studies, HFV based vectors replicated better than murine leukemia virus (MuLV)-based vectors in stationary-phase culture (Hirata *et al.*, 1996). Thus, gene transduction with FV vectors is likely to compare favorably to that with MuLV vectors. The use of FV has an additional advantage of being able to create replication-competent vectors that function with high efficiency in cell cultures. A *cis*-acting element in the SFV *pol* gene is required for genome packaging (Wu *et al.*, 1998). The need for this packaging signal sequence is unique for retroviruses, but whether the corresponding sequence of FFV has a similar requirement is unknown. The HFV Tas has the capacity for transactivating the HIV-1 LTR promoter (Löchelt *et al.*, 1994; Löchelt *et al.*, 1995). Furthermore, the HFV Tas may be capable of transactivating other viral, or even cellular, promoters. In contrast, there is no evidence that the FFV Tas interferes with either the human retroviral or cellular promoters. Thus, an FFV vector with the human *env* gene substituted for the feline *env* gene to promote replication in human cells may be a safe and useful vector for human gene therapy. The fact that there are a number of FFV field isolates is also great advantage for making variety of FFV-based vector constructs. The problem of immune rejection of a vector after repeated administration may be circumvented by the sequential use of different FFV field isolates as vectors.

AIMS AND SCOPE OF THE THESIS

Research on these interesting viruses, FFV, is rapidly expanding. There are many unanswered questions concerning the replication of FFV, some of which were described in this thesis. There are many challenging questions about the interaction of FFV with cellular and host defense mechanisms, which need to be pursued. A central question is why FFV, which is highly cytopathogenic in cell cultures, does not cause clinically apparent disease in either naturally or experimentally infected host. Identification of functions of regulatory genes such as *bel*-1 and *bel*-2 may be useful for understanding the mechanisms of pathogenicity. There is also a need to isolate FFV infectious DNA. If infectious DNA constructs can be made, it may be suitable for use in gene transfer experiments.

FIGURE LEGENDS

Fig. 1. Phylogenetic relationships of retroviruses. Alignment was done using the RT domain of Pol. EFV; equine foamy virus, BFV; bovine foamy virus, HFV; human foamy virus, FFV; feline foamy virus, HIV; human immunodeficiency virus, SIV; simian immunodeficiency virus type 1, MLV; Murine leukemia virus, MERV; endogenous viral element from mice, BLV; bovine leukemia virus, HTLV-1; human T-cell leukemia virus types 1, RSV; Rous sarcoma virus, MMTV; murine mammary tumor virus.

Fig. 2. Cytopathic effects of HFV. (A) Typical syncitia were formed in BHK cells infected with HFV. (B) Mock infected BHK cells.

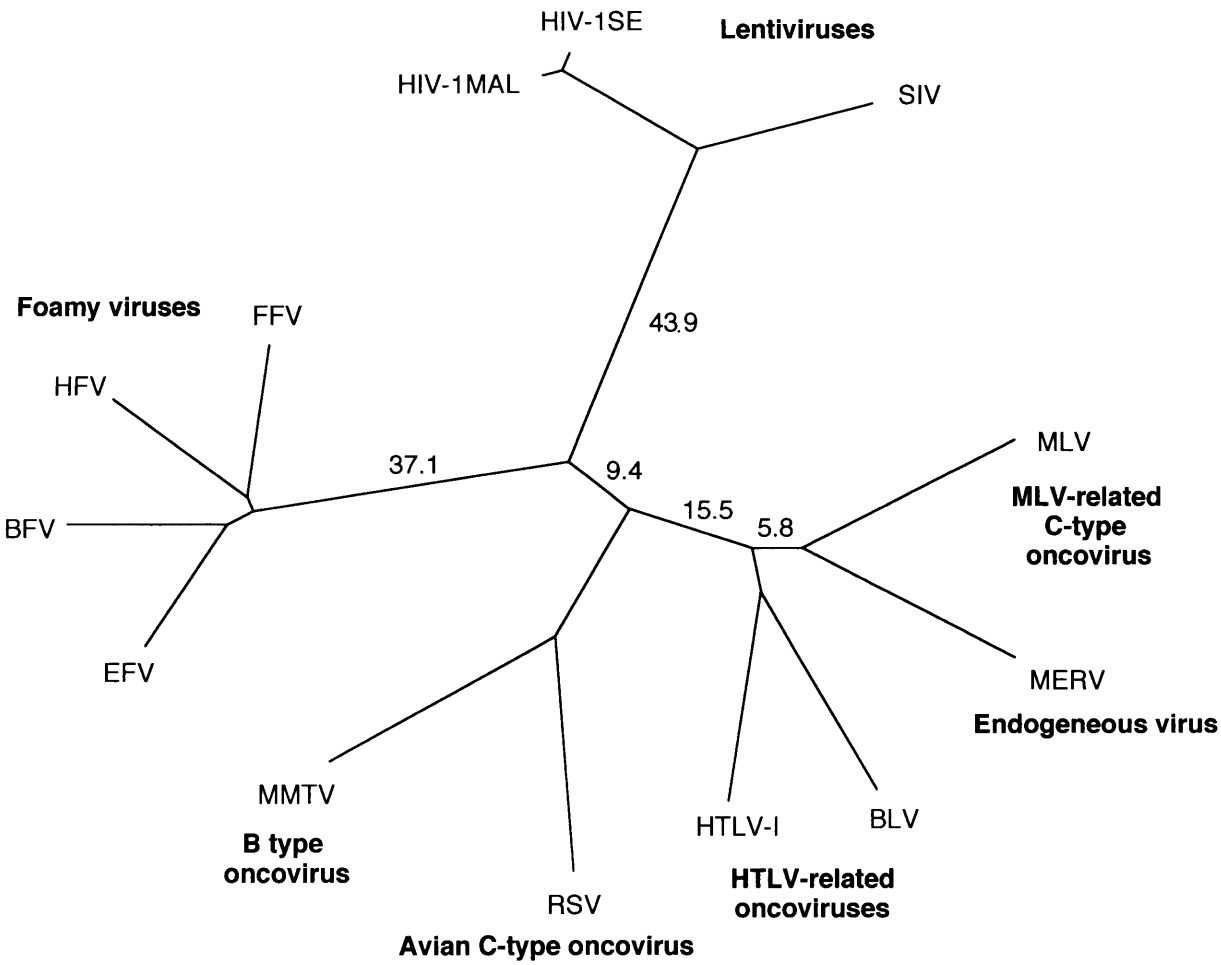
Fig. 3. Genome organization of FFV and other retrovirus genus. The organization and relative position of each ORF is shown as boxed region. ψ: packaging signal.

Fig. 4. Viral proteins encoded by FFV. An arrow shows the most efficient protease cleavage sites. Three Gag GR boxes are indicated by shaded boxes. The membrane spanning domain (MSD) in Env is indicated by a oblique lined box. DB, DNA binding domain; AD,

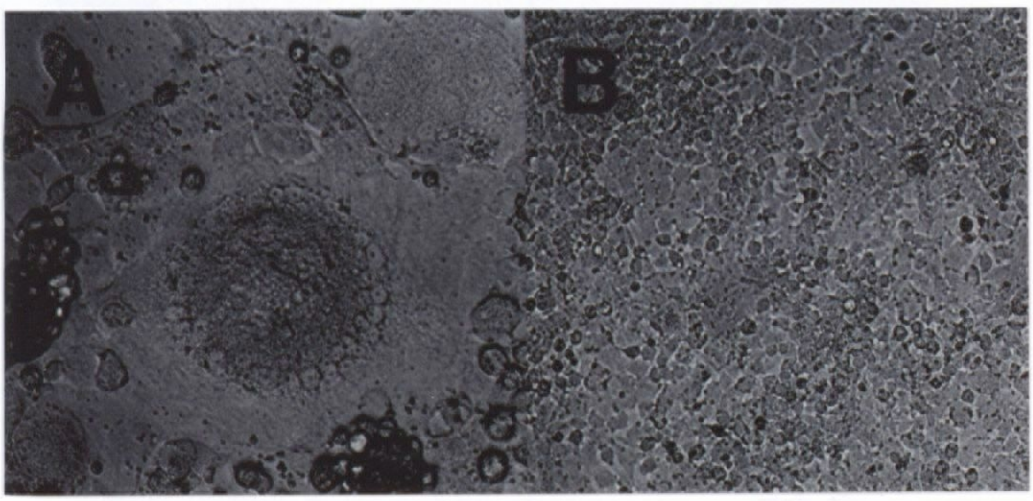
activation domain; NAB, nucleic acid binding; NLS, nuclear localization signal; ERS, endoplasmic reticulum retrieval signal.

Fig. 5. Replication pathway of FFV compared to that of conventional retroviruses and hepadnaviruses. (A) Proposed replication pathway of FFV. A large number of intracellular particles in infected tissue culture, which have been detected by both electron microscopy and viral assays, are shown. It is still obsecure whether such particles contain RNA, DNA or both. Uncertain steps in the life cycle are indicated by question marks. (B) Life cycle of lentiviruses. (C) Life cycle of hepadna viruses.

General introduction Fig. 1.



Genaral introduction Fig. 2.



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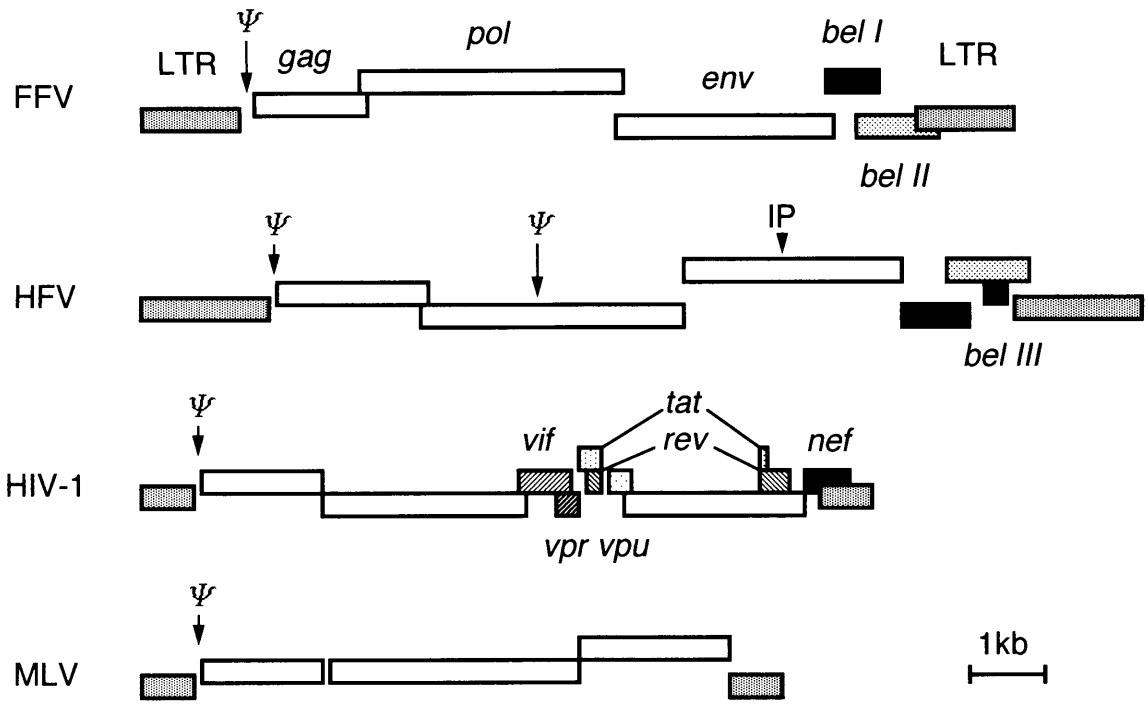

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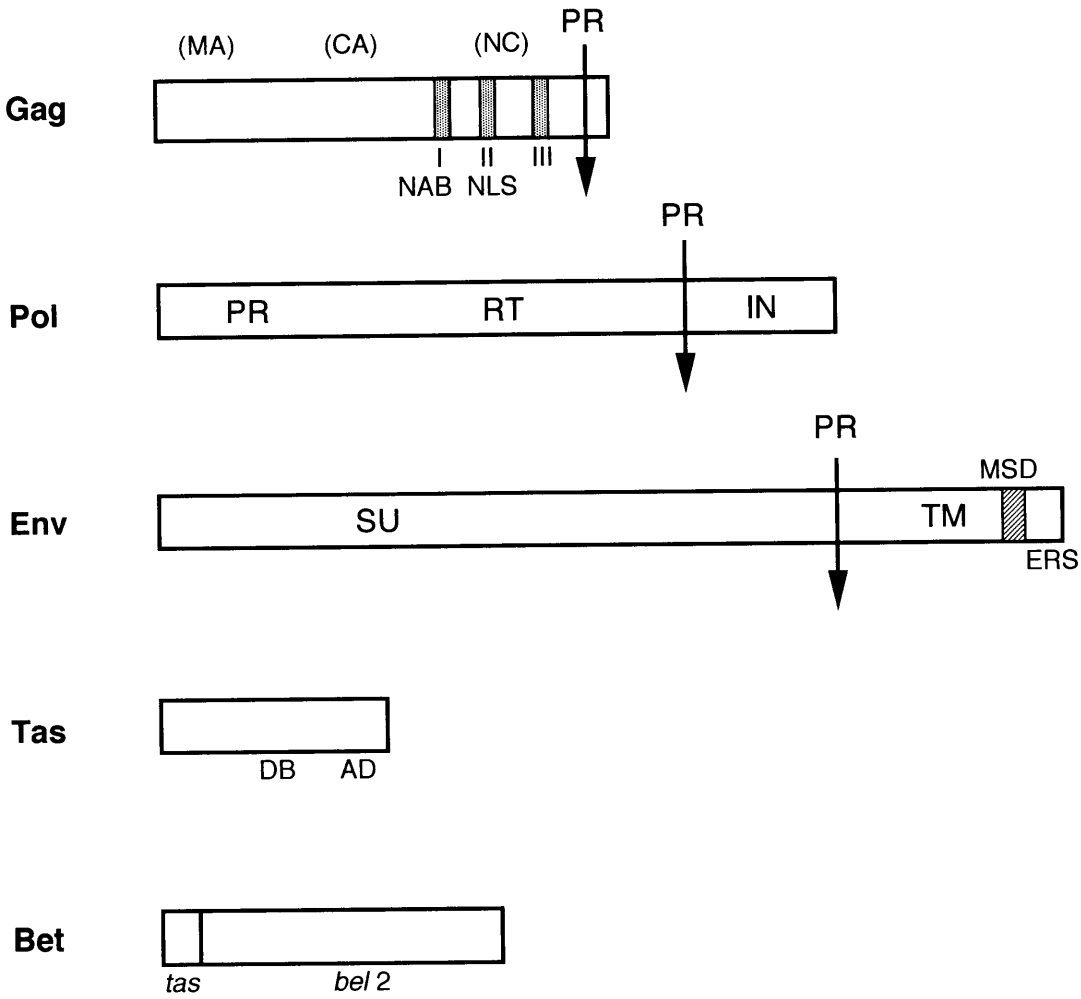

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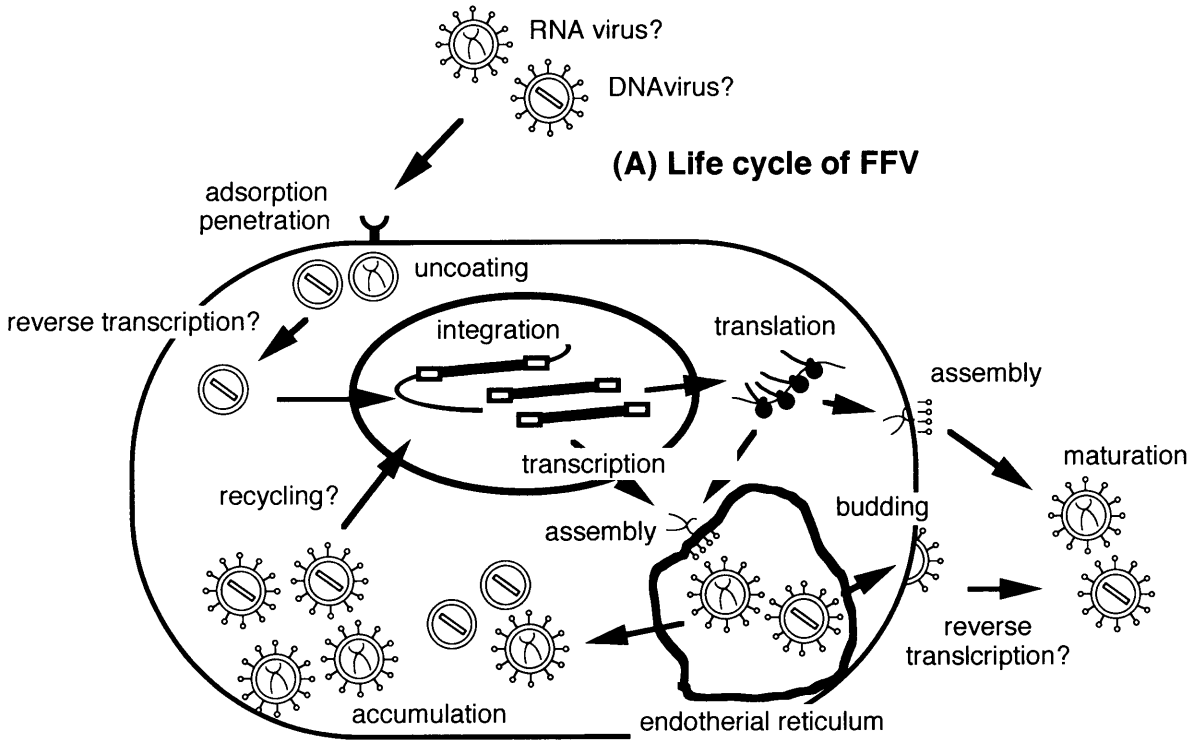
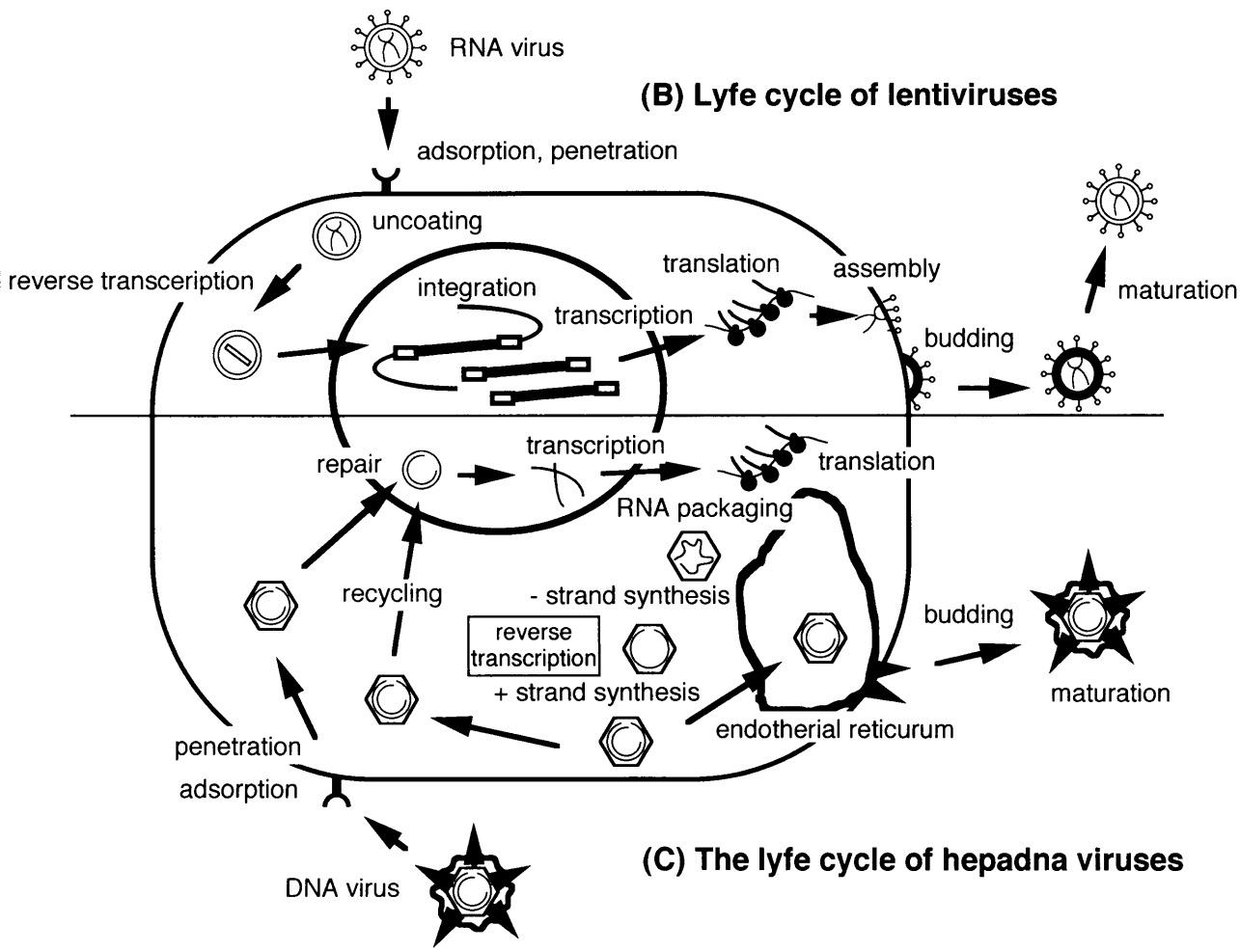




CHAPTER 1

Reactivation of feline foamy virus from a chronically-infected feline renal cell line by trichostatin A

ABSTRACT

Although acute infection of feline foamy virus (FFV) is normally highly cytopathogenic in Crandell feline kidney (CRFK) cells, a non-cytopathic persistent infection was established in the cells after cocultivation of the initially infected cells with uninfected cells at 4 times. To investigate reactivation of persistent infection, CRFK cells chronically infected with FFV were treated with trichostatin A (TA), a histone deacetylase inhibitor. TA induced higher FFV production from the Coleman strain carrier culture and also induced marked syncytium formation. In contrast, human foamy virus (HFV), which contains less homologous long terminal repeat (LTR) and putative internal promoter (IP) sequences, persistently infecting baby hamster kidney (BHK) cells were not reactivated by TA. The Sammy-1 strain of FFV, from which a part of the U3 region in the LTR is naturally deleted, showed less reactivation. The Coleman LTR promoter-based β-Gal expressing plasmid was activated in the persistently Coleman-infected cells in the presence of TA, whereas, the Sammy-1 LTR was not activated. Furthermore, the amounts of Gag protein expressed did not change in the presence or absence of TA. Because the putative IP region was very similar between these strains, the initiation by TA is relatively specific for LTR sequences, and therefore, histone deacetylation is at least in part responsible for reactivation of FFV from carrier cell culture.

INTRODUCTION

Foamy viruses (FVs) have been isolated from a large number of mammalian species (Malmquist *et al.*, 1969; Riggs *et al.*, 1969; Kennedy-Stoskopf *et al.*, 1986; Achong *et al.*, 1971; Hooks and Gibbs, 1975; Hruska and Takemoto, 1975; Flanagan, 1992). The feline foamy virus (FFV), which is classified as a member of the genus *Spumavirus* of *Retroviridae*, was first isolated in 1969 (Kasza *et al.*); several strains of FFV and FFV-related viruses have been reported since then (Riggs *et al.*, 1969; Mochizuki and Konishi, 1979; Ikeda *et al.*, 1997). Most of FVs are highly cytopathogenic leading to cell death *in vitro* (Kasza *et al.*, 1969; Mergia *et al.*, 1996; Mikovits *et al.*, 1996); however, paradoxically, persistently infected cells have been established *in vitro* without cell death (Miyazawa *et al.*, 1995). The mechanisms of persistent infection with FFV are not yet understood.

For FV transcription, Bel-1 protein is known to be an absolutely required as a transacivator (Tas), similar to Tat of HIV (Baunach *et al.*, 1993). A putative Tas-specific binding site was found in both the HFV LTR and IP, but the elements have little sequence identity (Kang *et al.*, 1998). Tas transcription is initially induced by IP (Yang *et al.*, 1997). Once the level of Tas is upregulated, the IP is switched to using LTR promoter for controlling transcription of the structural genes (Kang *et al.*, 1998). However, Tas is not found in viral particles. Thus, after chronic FV infection, the reactivation mechanism is still unknown.

Recent investigations have revealed that histone acetyltransferases are critical in activating and silencing promoter activities by altering the chromatin structure (Pfeffer and Vidali, 1991). Trichostatin A (TA) is a specific inhibitor of histone deacetylases (Yoshida *et al.*, 1990). TA has no effect on HIV-1 retroviral promoter activity when HIV promoter-reporter plasmids are unintegrated (Lint *et al.*, 1996). However, upon integration, TA activates HIV-1 transcription (Sheridan *et al.*, 1997) as well as human papillomavirus promoter activity (Zhao *et al.*, 1999) and expression of a transduced adenovirus promoter-*lacZ* gene (Chen *et al.*, 1997). These reports suggest that TA is a useful tool to highly express some transduced genes. Early studies, in which FVs were applied as gene delivery vehicles (Bieniasz *et al.*, 1997; Schmidt and Rethwilm, 1995; Russell and Miller, 1996), were carried out despite the fact that the infectious virus titers were marginal (Schmidt and Rethwilm, 1995; Bieniasz *et al.*, 1997); however, as yet, there have been no studies of whether TA regulates the FV promoter.

Because of the proposed use of FFV vectors for gene therapy (Bieniasz *et al.*, 1997) and elucidation of initiation of FV infection (Yu *et al.*, 1996-a), it is of interest to determine whether treatment with TA could be responsible for the enhancement of FFV replication. In this study, the author therefore examined whether TA can break the persistent state of FFV-infected cells and also considered which factors may be related to chronicity of FFV infection.

MATERIALS AND METHODS

Viruses and cells

Three FFV strains, American isolate Coleman (Ikeda *et al.*, 1997), and Japanese isolates S7801 (Mochizuki and Konishi, 1979) and Sammy-1 (Miyazawa *et al.*, 1995), were obtained as described previously. The HFV strain, HS007 (Adachi *et al.*, 1995) was a kind gift of Dr. A. Adachi, The University of Tokushima School of Medicine. Reverse transcriptase (RT) non-producing cells derived from CRFK (ATCC CCL 94) (Crandell *et al.*, 1973), designated NV112, were subcloned and used for CPE and β–Gal assays. CRFK cells persistently infected with FFVs were established as described previously (Miyazawa *et al.*, 1995), and were cultivated in Iscove's modified Dulbecco's medium (IMDM) (Gibco. Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics.

Viral titration

Two-fold serial dilutions of the culture media (100 μl) from the infected cells were added to cells seeded in a 96-well plate as described previously (Ikeda *et al.*, 1997). After cells were passaged two times at six day intervals, infection by FFV was judged by the CPE observed by microscopy. The virus titers are presented at the 50% tissue culture infective dose ($TCID_{50}$).

RT assay

Virion-associated reverse transcriptase (RT) activity was measured with 2.5 mCi of [methyl-^{3}H]-deoxythymidine 5'-triphosphate with 2 mM Mn^{2+} by the method of Johnson *et al.* (Johnson *et al.*, 1989).

Plasmid constructs and β-Gal assay

The LTR region was amplified from total DNA extracted from FFV carrier cell cultures by PCR (primer F, TCCAAGTGATGTTGCTTCCC; and primer R, CAGTAAGGTAAGGTCCCTAG) using LA-Taq polymerase (Takara, Kyoto, Japan), and inserted into the pCR2.1 TA cloning vector (pCR/LTR1 of Coleman, pCR/LTR2 of S7801 and pCR/LTR3 of Sammy-1). The DNA was automatically sequenced by M13 primer using a DSQ-2000L machine (Shimazu, Tokyo, Japan). The sequences of Coleman, S7801 and Sammy-1 LTRs described here have been submitted to DNA Data Bank of Japan with the following accession numbers; AB042566, AB042567 and AB042568, respectively. The accession number of the FUV LTR used here is Y08851. FFV enhancer and promoter-directed β-Gal expression plasmids (pSV/LTR-1, pSV/LTR-2, pSV/LTR-3) were constructed by introducing the 1.4-kb Eco RI (blunted) fragment of pCR/LTR1-3 into pSV-β-gal digested with Hind III (blunted). All plasmids were prepared for β-Gal assays by alkaline lysis and purification by cesium chloride centrifugation. Transient transfection into the FFV carrier cells was performed using the Lipofectin liposomal transfection reagent as specified by the manufacturer (Life Technologies,

Germany). Briefly, subconfluent cells in 60-mm diameter plates were transfected with 5μg of total plasmid DNA and 25μl of Lipofectin (Gibco) as described previously (Fujii *et al.*, 1996). For β-Gal assays, cells were fixed in 0.5% glutaraldehyde and stained with 2% X-Gal (Takara, Kyoto, Japan) substrate with 50mM potassium ferri/ferro-cyanide buffer. Specific activation of LTR was calculated as follows; specific activity of β-Gal= positive cell numbers of experimental sample – spontaneously positive cell number of control sample. Spontaneous activation was measured by transfection of LTR-β-Gal plasmids alone into uninfected CRFK cells. The values shown are the means ±SD of at least three independent transfections.

Profiles of transcription factor binding sites were analyzed by TFSEARCH ver. 1.3 in GenomeNet web site.

Immunoblotting

Electrophoresis was performed in 10% polyacrylamide gels containing 0.1% SDS. After transfer to polyvinylidene difuluoride membranes (Millipore, USA, MA), the blotted proteins were blocked with 5% dry milk in PBS with 0.1% NP-40 overnight. Anti-FFV and control feline sera obtained from an FFV-infected and a specific pathogen-free (SPF) cats were used as a first antibodies, and anti-cat IgG conjugated to the alkaline-phosphatase (Boehringer-Mannheim, Germany) was used as the secondary antibody. The membrane was stained as described previously (Otake *et al.*, 1994).

Electron microscopic analysis

FFV-infected and uninfected cells were washed and fixed in 2% glutaraldehyde followed by 0.1 M phosphate buffer (pH 7.2) containing 2% osmium tetroxide. Samples were then stained with 1% uranyl acetate, dehydrated with a graded series of ethanol dilutions followed by 100% ethanol propylene oxide, and embedded in epoxy resin. Thin sections were cut and stained with lead citrate. The sections were viewed and photographed using a JEOL model 1200 EX.

RESULTS

Reactivation of FFV Production from Carrier Cell Culture by Treatment with TA.

To determine whether the inhibition of histone deacetylation was the cause of reactivation of FFV, three FFVs (Coleman, S7801 and Sammy-1 strains) and HFV carrier CRFK and BHK cells, respectively, were treated with various amounts of TA for 3 days (0.3 nM to 3 μM). Viral titers in the culture supernatant were determined as 50% tissue culture infective dose ($TCID_{50}$) by the cytopathic effect (CPE) assay. Although the viral titer was initially low in the media of carrier culture, the TA treatment raised the viral production from the Coleman-carrying cells in a dose-dependent manner (Table 1). However, this reagent was not so effective against S7801, Sammy-1 (Table 1) and HFV (data not shown).

Furthermore, after the treatment with TA, CRFK cells persistently infected with Coleman strain of FFV were stained with May-Grünwald-Giemsa and were examined microscopically. The morphology of carrier cell cultures was dramatically changed by TA treatment. TA-treated Coleman carrier culture exhibited marked a CPE with large syncytia (Fig. 1D, E, and F), very different from cultures not treated with TA (Fig. 1C) or control CRFK cells (Fig. 1A and B). However, the author could not find any morphological changes in S7801 and Sammy-1 carrier cultures (data not shown).

The author also examined the effect of TA treatment by reverse

transcriptase (RT) assays as described in Materials and Methods. Virion-associated RT activity in the Coleman carrier culture supernatant increased from 850±10 to 1,433±21 dpm with the treatment of 3 μM TA, while RT activity of S7801 and Sammy-1 did not show any significant increase (from 603±40 to 846±36, and from 520±26 to 487±30, respectively). As a negative control, RT activity in the supernatant of CRFK culture was 64±4 dpm.

To compare the effect of other reagents on viral reactivation, the cells were treated with 5'-azacytidine, which has been used to reactivate endogenous genes whose promoters and enhancers are methylated (Ferguson *et al.*, 1995; Lee *et al.*, 1995; Yoshiura *et al.*, 1995). This reagent slightly reactivated viral production from all lines (Table 2). However, the reactivity is rather low compared with TA treatment of the Coleman strain. In contrast, treatment with sodium butyrate, which inhibits histone deacetylases and has a number of other activities (Kruh, 1982), did not reactivate viral production at a concentration of 50 mM (Table 2). Initial tests performed with a broad range of sodium butyrate concentrations yielded similar results (data not shown). These results suggest that the mechanism for reactivation of viral transgenes may be different from the activation of some endogenous genes. Thus, histone acetylation is likely to direct viral production in the initial phase of replication in the persistently-infected cells, but sensitivity to the acetylase reagent is different among the three viral

strains.

Partial Deletion of U3 in the LTR Sequence of FFV

The proviral nucleotide sequence of the FFV long terminal repeat (LTR) region was analyzed and compared among three different FFV strains (Coleman, S7801 and Sammy-1) aligned with the sequence of FUV prototype strain (Fig. 2). The Coleman LTR was 1,390 nucleotides long, and corresponds to the homologous region in FUV strain. Homologies of nucleic acid sequences of Coleman LTR to FUV and to HFV LTR were 97.7 and 47.1%, respectively. On the other hand, S7801 and Sammy-1 LTRs were different from the FUV LTR in length (1,372 and 1,371). Although the putative transcriptional factor binding motifs, such as heat shock transcription factor (HSF) and AP-1 transcription factor, are conserved in the LTR promoter region, a deletion in the U3 region of S7801 and Sammy-1 (from nt 615 to nt 641) was found (Fig. 2). The nucleotide sequences of putative internal promoter regions among the three strains were also examined, but significant differences were not found (data not shown). The HFV LTR did not contain homologous DNA sequences corresponding to the deleted region in U3 of S7801 and Sammy-1 LTR. From these data, it is concluded that FFV reactivation by TA treatment is LTR-specific and dependent on this deleted promoter element.

To investigate reactivity of Tas against the LTR promoter caused by TA treatment, β-galactosidase (β-Gal) assays were performed. Three LTR-

reporter plasmids derived from Coleman, S7801 and Sammy-1 FFV strains were transiently transfected into FFV carrier cells. TA intensively induced high FFV promoter activity of Coleman LTR-β-*gal* reporter in Coleman carrier cultures. In contrast, LTR of the other two strains did not (Table 3). S7801 and Sammy-1 LTRs were slightly activated by TA in their own virus carrier cultures (Table 3). Control pSV promoters were not activated by Tas in the presence or the absence of TA (Table 3). These data suggest that Tas is crucial to viral transcription and that TA enhances the transactivation in an LTR-dependent manner.

Gag Expression and Morphology of Viral Structure in FFV

Viral protein expression in the carrier culture with or without TA treatment was examined by immunoblotting (Fig. 3). The lysates of three FFV carrier cells were subjected to SDS-PAGE, transferred to membranes, and stained with FFV-infected cat serum. An approximately 52 kDa band corresponding to Gag was observed in all of the carrier cells. The CRFK cells as a negative control, did not show any positive bands (Fig. 3). Unexpectedly, expression levels of Gag proteins in the presence of TA (Fig. 3, lane 3 of Coleman, lane 5 of S7801 and lane 7 of Sammy-1) were the same as in its absence (Fig. 3, lane 4 of Coleman, 6 of S7801 and 8 of Sammy-1). Bands of approximately 48 kDa bands which were thought to be a processed Gag protein were also found in the Coleman and S7801 carrier cells, but not in Sammy-1 (Fig. 3).

Electron microscope (EM) was used to examine the budding of FFV particles from the plasma membrane after treatment with TA. The numbers of budded Coleman and S7801 particles were increased to hundreds of virions per electron microscopic area by TA-treatment, but this was not observed in cells infected with the Sammy-1 strain. Cells infected with Coleman virus accumulated intracellular naked viral capsids (Fig. 4). Interestingly, Coleman particles were contained spike-like structures, probably representing envelope protein, whereas S7801 and Sammy-1 possessed multimembrane-enclosed particles with no spike appearance, containing an electron-dense viral core (Fig. 4). These particles were not observed in the control CRFK cells (Fig. 4).

DISCUSSION

In this chapter, the author shows that TA treatment induces viral production at a higher-titer from the carrier culture of FFV/CRFK. Chen *et al.* (1997) have recently demonstrated that the treatment with 5-azacytidine and sodium butyrate significantly increased reactivation of adenovirus promoter-*lac* Z gene expression. In addition, both human and simian FVs have been reported to be reactivated by 5-azacytidine (Hoota and Loh, 1987; Schweizer *et al.*, 1993) and TA reactivates HIV-1 transcription (Sheridan *et al.*, 1997). Although butyrate showed less reactivation compared with TA, these data suggest that histone acetylation is important for reactivation of FFV.

The Japanese isolates, S7801 and Sammy-1, contained deletions in the 5' LTR promoter U3 regions as compared with the American isolate, Coleman (Fig. 2). HFV variants with the largest U3 deletion showed low ability to replicate in human cell cultures (Schmidt *et al.*, 1997). In addition, the nucleic acid sequences of the putative IP region and the U5 region of the FFV and HFV LTR were conserved (Kang *et al.*, 1998), suggesting that deletion of the U3 promoter region is related to the reactivation of FFV *in vitro*.

The Bel-1 protein has been reported to be an essential requirement for LTR- and IP-mediated FV transcription (Baunach *et al.*, 1993). The results in this chapter strongly support the view that the presence of Bel-1 is

absolutely necessary for LTR-mediated transcription. Interestingly, the level of expression of Gag was not altered by treatment with TA, and no difference in the amounts of Gag mRNA was detected by semi-quantitative RT-PCR (unpublished data). Further, in the presence or absence of TA, Hirt's extracted DNA copies (Hirt, 1967) of Coleman carrier cells were equivalent to those of S7801 carrier cells. These data suggest that TA-treatment has no effect on the accumulation of unintegrated FFV DNA, and that TA could only change initiation of Bel-1 production. It has been reported that Bel-1-defective forms of HFV proviruses can prevent cell lysis and lead to persistent infection (Saib *et al.*, 1993). Histone acetylation by TA is likely to direct initiation of Bel-1 expression and LTR deletion may be related to this initiation. Thus, initiation of FFV reactivation by TA may involve the deleted element of the U3 promoter. Investigations of the precise region spanning the putative regulator element are currently underway.

In the case of the Sammy-1 strain, Gag protein did not appear to be efficiently cleaved. Because it has been suggested that processing of Gag has a critical role in infecting host cells, infection of progeny virus to new cells *in vitro* may be related to Gag cleavage by protease. Therefore, viral particles might have been rarely detected in persistently infected cells in EM analysis. Although spike-less alteration of FV morphology has been observed in chimeric FVs (Pietschmann *et al.*, 1999), the results in this chapter indicate the possibility of an alteration in the viral construct of Sammy-1. The morphological alteration is likely to cause incomplete

cleavage of Gag protein and the uncleaved Gag may also contribute to the persistent state via a lesser infectivity of viral particles; however, more precise examination needs to be done to define viral capsid construction.

FIGURE REGENDS

Fig. 1. Morphological alterations of FFV infected CRFK cells caused by TA treatment. CRFK cells persistently infected with Coleman (C to F) or mock-infected (A and B) were treated with TA for 3 days at a concentration of 3 μM (B, D and F) or 0.3 nM (E) or were left untreated (A and C). After May Grünwald-Giemsa staining, cells were examined microscopically. Magnifications: A to E, ×100; F, ×200.

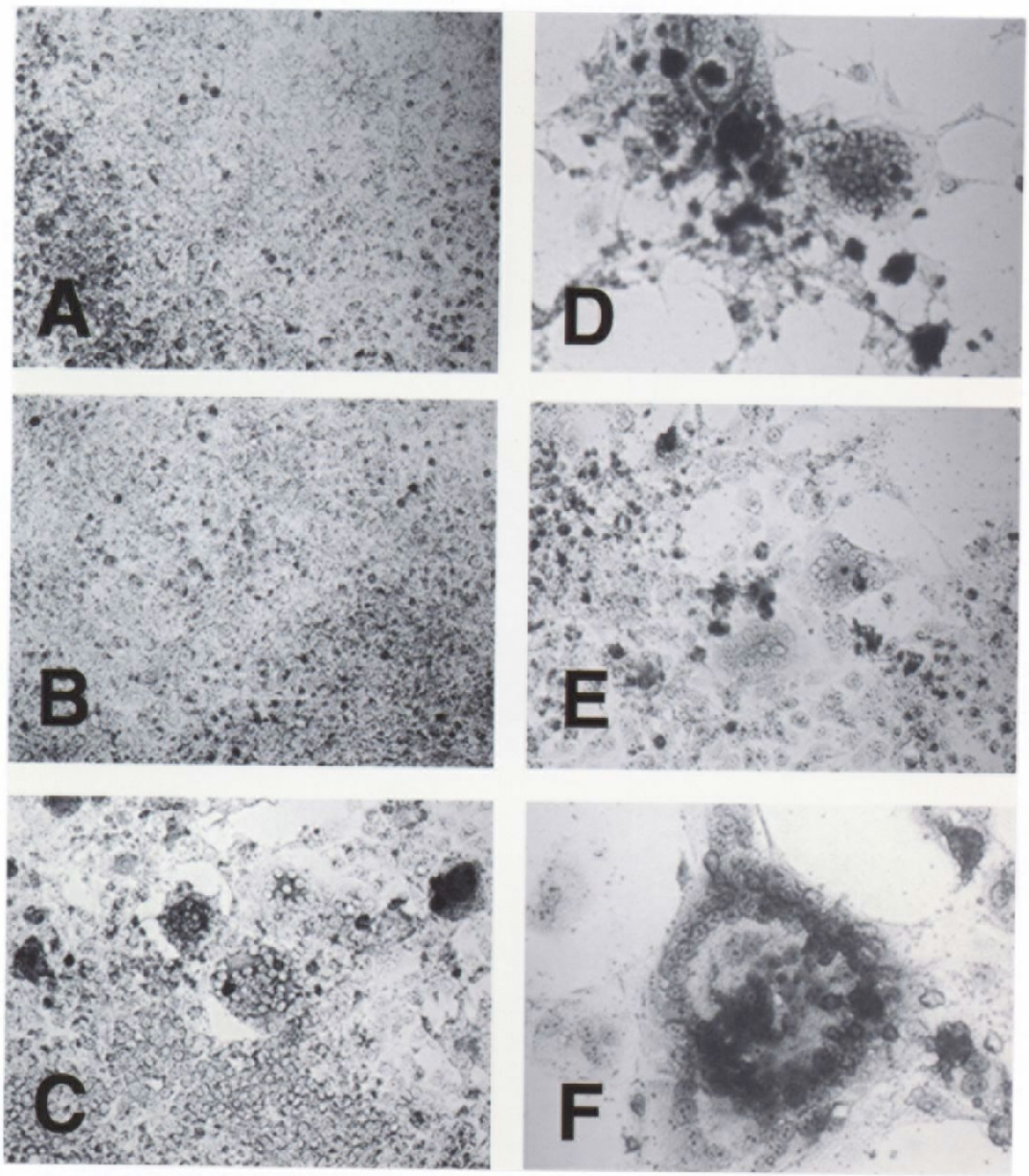
Fig. 2. Genomic organization and alignment of the nucleotide sequence of the promoter-enhancer region in FFV. The complete 5' LTR sequences among three FFV strains (Coleman, S7801 and Sammy-1) are presented with the prototype FFV FUV sequence. The scale at the top is drawn with respect to the start of the U3 region of the 5' LTR. Numbers in the alignment of the sequences represent distance along the genome in base pairs. Deletions in the U3 promoter region in the 5' LTR are boxed. The end of U3, R and U5 regions are represented by solid triangles with bars. Transcriptional factor binding motifs in the promoter region are marked as solid arrows.

FIG. 3. Detection of Gag-specific proteins in FFV carrier cells. CRFK cells persistently infected with Coleman (lanes 3 and 4), S7801

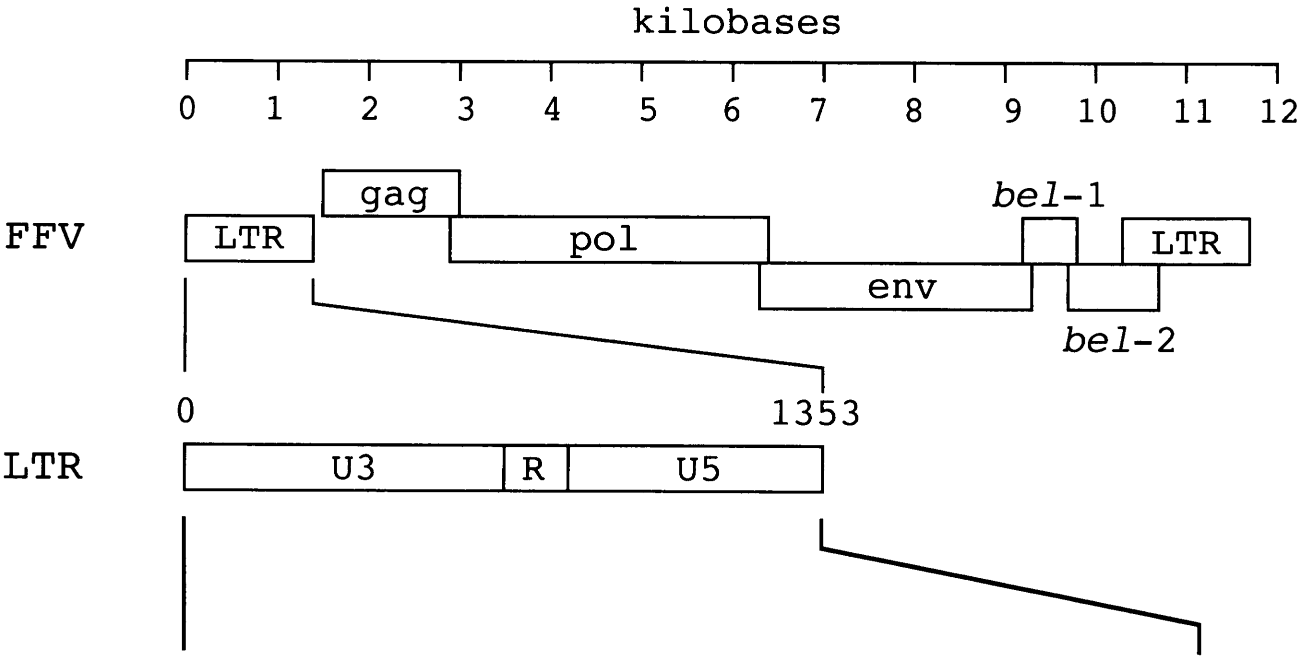
(lanes 5 and 6), or Sammy-1 (lanes 7 and 8) strains, or mock-infected (lanes 1 and 2) were treated with TA or left untreated. After three days incubation, cells were harvested, electrophoresed by SDS-PAGE, and analysed by immunoblotting with FFV Coleman-infected cat serum. The molecular masses of proteins are given on the left margin.

FIG. 4. Electron micrographs of FFV persistently-infected CRFK cells. FFV Coleman, S7801 and Sammy-1 carrier cells were treated with 50 μM of TA or were untreated. After 3 days, cells were fixed and then were subjected to electron microscopy. Bar size; 200 nm (FFV-infected cells) and 1 μm (Control)

Chapter 1. Fig. 1.



Chapter 1. Fig. 2.

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                                                                                                  HSF
FUV        1 TGTCATGGGCCAAAGAGAATTCTCACAGAGGAGAATACTCTCTGCTGCCATCTAGTGACGATGAGGAAGAAGAAATGTCAGAAAGAGAGG   90
Coleman    1 TATCATGGGCCAAAGAGAATTCTCACAGAGGAGAATACTCTCTGCTGCCATCTAGTGACGATGAGGACGAAGAAATGTCAGAAAGAGAGG   90
S7801      1 TGTCATGGGCTGAAGAGAATTCTCAGAGAGGAGAATACTCTCTGCTGCCATCTAGTGATGATGAAGAAGAAGAAATGTCGGAGAGGGAGG   90
Sammy-1    1 TGTCATGGGCTGAAGAGAATTCTCAGAGAGGAGAATACTCTCTGCTGCCATCTAGTGATGATGAAGAAGAAGAAATGTCGGAGAGGGAGG   90
             *.********  ************* ******************************** ***** **.*********** ** ** ****

FUV       91 AATTATTGTGCCATATAAATCAGTGTCAACAAAAGCTCTTTTATCCCGGAGGGACGACTGATGTCCTTGGAATGGAAAGCAATGTTTGGC  180
Coleman   91 AATTATTGTGCCATATAAATCAATGTCAACAAAAGCTCTTTTATCCCGGAGGGACGACTGATGTCCTTGGAATGGAAAGCAATGTTTGGC  180
S7801     91 AATTATTGTGTCATATAAATCAATGTCAACAAAAGCTCTTTTATCCCGGA-GGACCACTGATGTCCTTGGAATGGAGAGCAATGATTGGC  179
Sammy-1   91 AATTATTGTGTCATATAAATCAATGTCAACAAAAGCTCTTTTATCCCGGA-GGACCACTGATGTCCTTGGAATGGAGAGCAATGATTGGC  179
             ********** ***********.************************** **** ******************** ******* *****

                                                                             HSF
FUV      181 TCACTAAATTTGTTAATATTAAATTTCCTAAAGGAACAAAAGTGATACTTCCTGATGGAAGAAAATTCATAGCCTGTGATCCTGAGCTAA  270
Coleman  181 TCACTAAATTTGTTAATATTAAATTTCCTAAAGGAACAAAAGTGATACTTCCTGATGGAAGAAAATTCATAGCCTGTGATCCTGAGCTAA  270
S7801    180 TTACTAAATTTGTTAATACTAAATTTCCTAAAGGAACAAAAGTGATACTTCCTGACGGAAGAAAATTTATAGCCTGTGATCCTGAGCTAA  269
Sammy-1  180 TTACTAAATTTGTTAATACTAAATTTCCTAAAGGAACAAAAGTGATACTTCCTGACGGAAGAAAATTTATAGCCTGTGATCCTGAGCTAA  269
             * ***************** ************************************ *********** *********************

                                                 AP-1 like
FUV      271 AACCATTATTGCAGGAATTGAAATTCTTGGATAGGGCAACATCTGAGTCATCTGACTCTGAATAGAAAGCCTGAATTTACCTGGATTATG  360
Coleman  271 AACCATTATTGCAGGAATTGAAATTCTTGGATAGGGCAACATCTGAGTCATCTGACTCTGAATAGAAAGCCTGAATTTACCTGGATTATG  360
S7801    270 AGCCATTATTGCAGGAATTGAAGTTCTTGGATAGGGCAACATCTGAGTCATCTGACTCTGAATAGAAAGCCTGAATTTACCTGGATTGTG  359
Sammy-1  270 AGCCATTATTGCAGGAATTGAAGTTCTTGGATAGGGCAACATCTGAGTCATCTGACTCTGAATAGAAAGCCTGAATTTACCTGGATTGTG  359
             * ******************** ******************************************************************* **

FUV      361 CAACTTTGTCCGAGGTGGCAGAGTGGTTATGTATCTGTCATACTCGGGGAAAGTTTTGTCTTTACATGTTCAAGACATATAAAGGGTGGA  450
Coleman  361 CAACTTTGTCCGAGGTGGCAGAGTGGTTATGTATCTGTCATACTCGGGGAAAGTTTTGTCTTTACATGTTCAAGACATATAAAGGGTAGA  450
S7801    360 CAACTTTGTCCGAGGTGGCAGAGTGGTTATGTATCTGTCATACTCGGGGAAAGTTTTGTCTTTACATGTTCAAGACATATAAAAGGTAGA  449
Sammy-1  360 CAACTTTGTCCGAGGTGGCAGAGTGGTTATGTATCTGTCATACTCGGGGAAAGTTTTGTCTTTACATGTTCAAGACATATAAAAGGTAGA  449
             ************************************************************************************* ***.**

FUV      451 AAAATATATTCCTGACTAAACTTCCTGGGGACTAGAGGTGTGGAAACTTTGCTGCCTCTGCTTCACGGGAAGTTTTTGGTTCGAATCCTT  540
Coleman  451 AAAATATATTCCTGACTAAACTTCCTGGGGACTAGAGGTGTGGAAACTTTGCTGCCTCTGCTTCACGGGAAGTTTTTGGTTCGAATCCTT  540
S7801    450 AAAATATATTCCTGACTAAACTTCCTGGGGACTAGAGGTGTGGAAACTTTGCTGCCTCTGCTTCACGGGAAGTTTTTGGTTCGAATCC-T  538
Sammy-1  450 AAAATATATTCCTGACTAAACTTCCTGGGGACTAGAGGTGTGGAAACTTTGCTGCCTCTGCTTCACGGGAAGTTTTTGGTTCGAATCC-T  538
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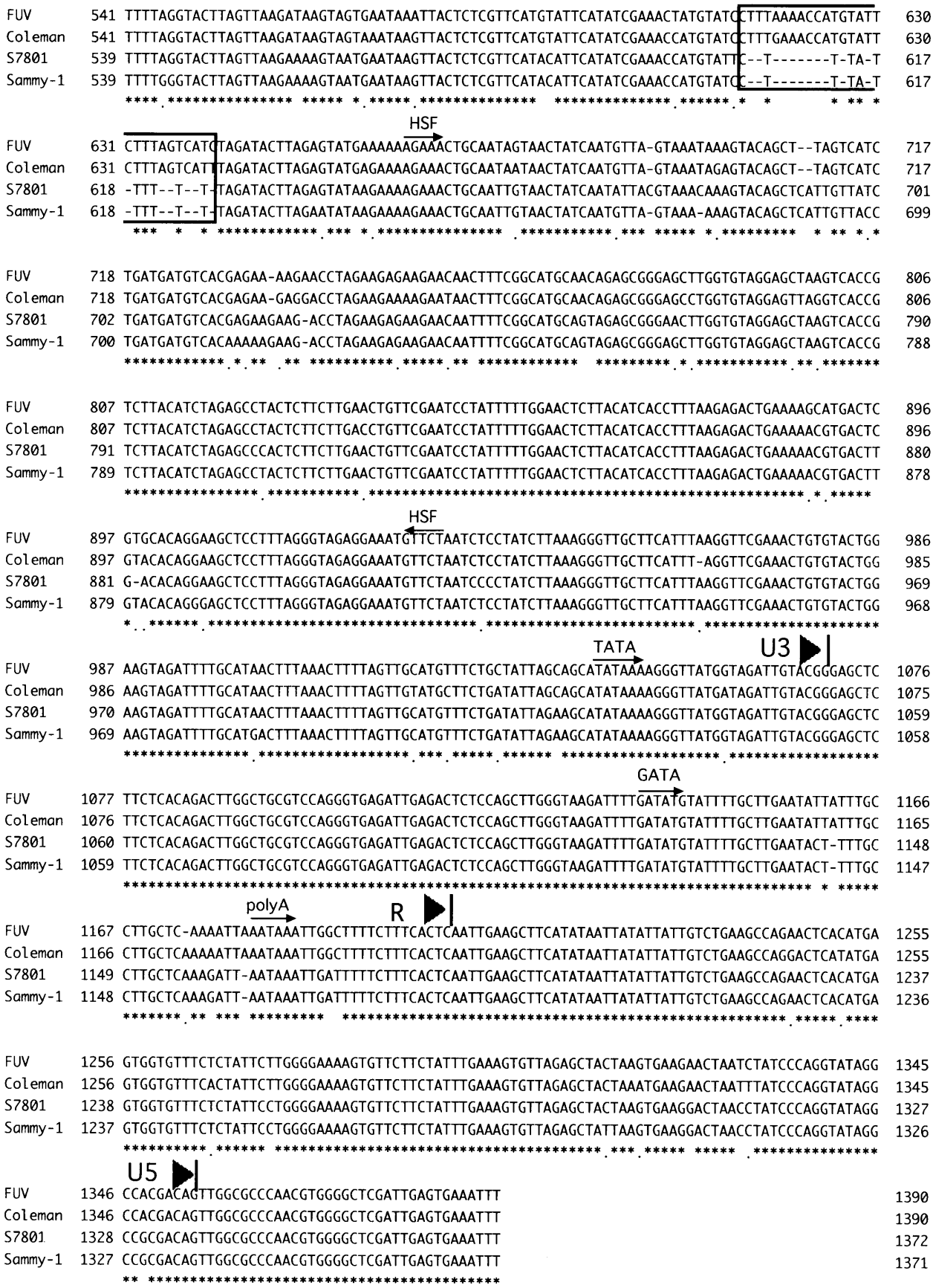
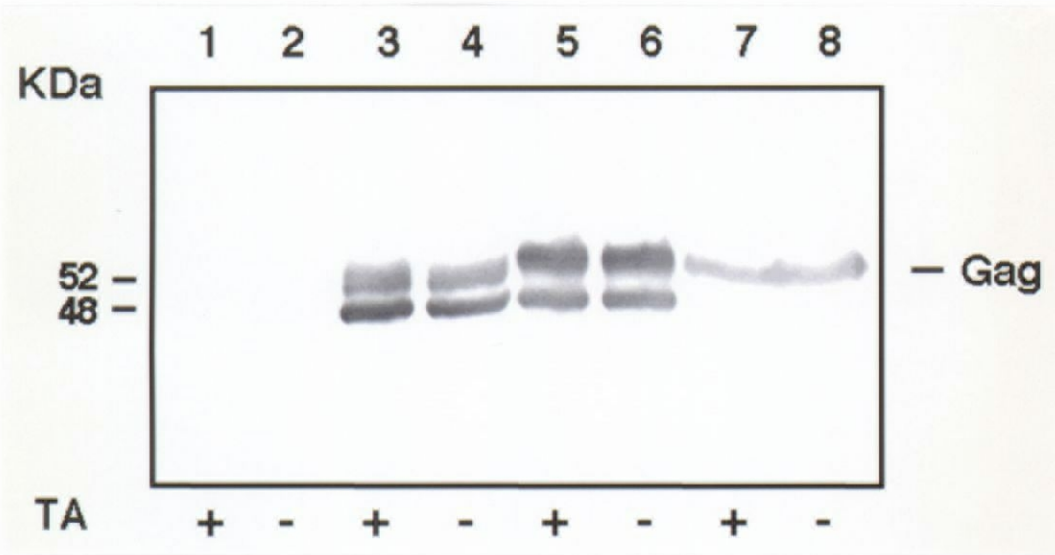
Chapter 1. Fig. 2. continued



Chapter 1. Fig. 3.



Chapter 1. Fig. 4.

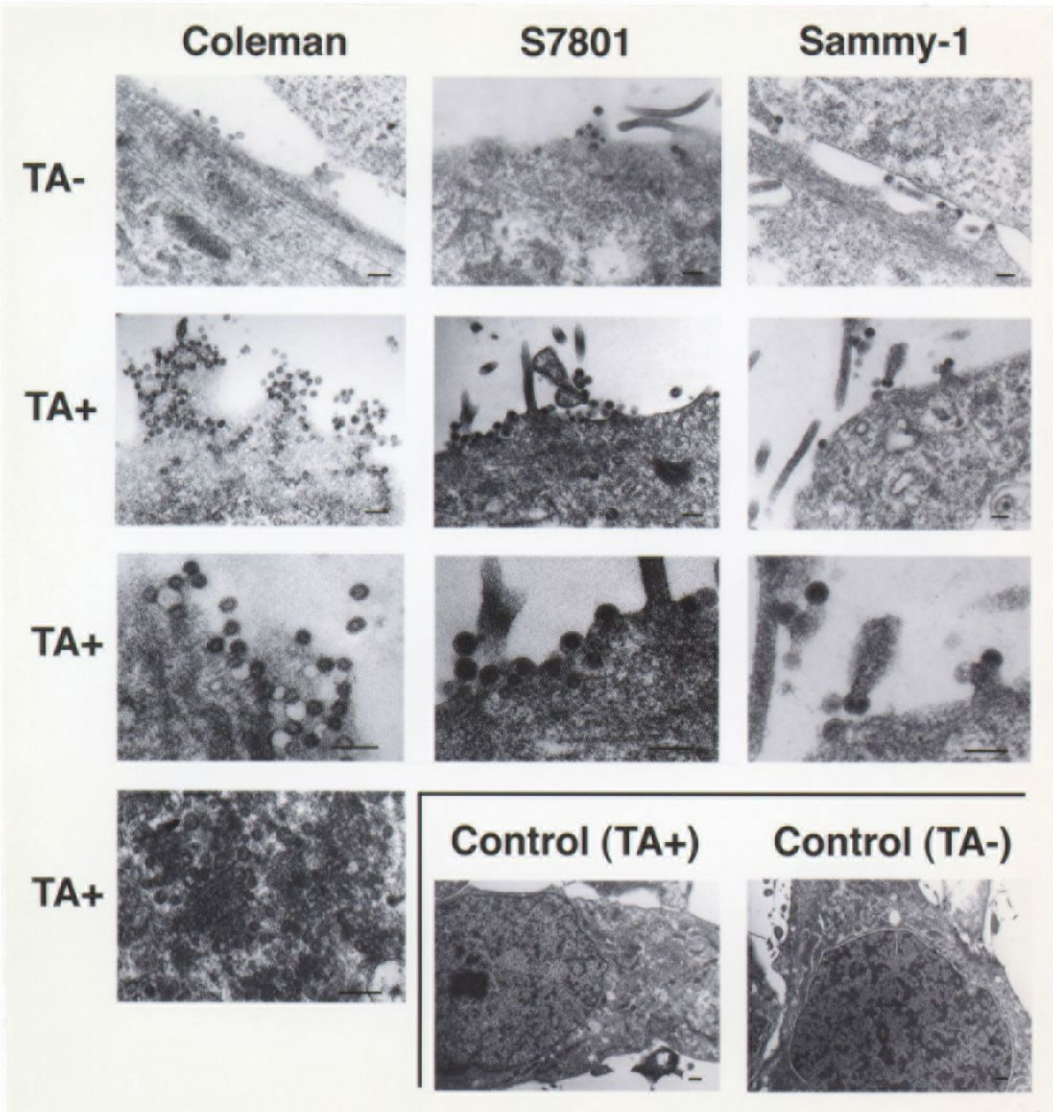


Table1 Reactivation of FFV strains in CRFK cells by treatment with TA

TA dilution	Persistent infection			Acute infection
	Coleman	S7801	Sammy-1	Coleman
10^{-4}	4.5±0.3	1.5±0.1	1.3±0.3	NT
10^{-5}	2.3±0.2	1.0±0.2	1.2±0.3	NT
10^{-6}	1.2±0.2	1.0±0.3	0.8±0.1	NT
10^{-7}	1.2±0.4	1.0±0.3	0.7±0.2	NT
10^{-8}	1.0±0.2	0.8±0.2	0.8±0.2	NT
0	0.8±0.1	0.8±0.2	0.7±0.2	2.5±0.2

The experiment was repeated at three times. Values are represented as mean virus titers by $\log_{10}TCID_{50}$/ml.
10^{-4} = 3μM TA concentration, NT; not tested.

Table 2 Reactivation of FFV in persistently infected cells after treatment with 5-azacytidine and sodium butyrate

Virus strain	5-Azacytidine	Butyrate	None
Coleman	1.8±0.2	0.8±0.1	0.8±0.1
S7801	1.8±0.3	0.7±0.2	0.8±0.2
Sammy-1	1.5±0.2	0.8±0.2	0.7±0.2

The experiment was repeated at three times. Values are presented as mean virus titers by $\log_{10}$ $TCID_{50}$/ml. Concentrations of 5-Azacytidine or Butyrate are 3 μM or 50mM, respectively.

Table 3 Activation of FFV LTR promoter-based *β-gal* reporter plasmids on FFV/CRFK cells by treatment with TA.

	β-Gal expressing plasmids			
Trans activator	Coleman-LTR	S7801-LTR	Sammy-1-LTR	pSV-βgal
Coleman	104±6 / 68±10*	44±8 / 87±2	89±5 / 80±4	0 / 0
S7801	56±2 / 35±8*	28±4 / 20±3	25±3 / 29±3	0 / 0
Sammy-1	17±2 / 14±3	8±1 / 14±1	15±1 / 9±1	0 / 0

The number of positive cells were counted by microscopic analysis after β-galactosidase staining . TA+ / TA-,
*P<0.01

CHAPTER 2

Comparison of feline foamy virus gene expression during acute and persistent infection in Crandell feline kidney cells

ABSTRACT

Although feline foamy virus (FFV) infection in cats is persistent, FFV is highly cytopathogenic in cell cultures. In the previous study, the persistent infection in a feline epithelial cell line (CRFK cells) has been established (Miyazawa *et al.*, 1995). To examine the mechanism of the persistent infection, the author compared both transcription and translation of virus genes during acute and persistent infections in the cells. The mRNAs transcribed by the internal promoter (IP) and the Bet and Bel-2 proteins were detected in the acute infection. On the contrary, in the persistent infection, transcription of these mRNAs was very weak and no expression of Bet and Bel-2 were observed. After the treatment of persistent cell culture with histone acetylation reagent, trichostatin A, transcription pattern did not alter. Therefore, suppression of the IP may play an important role for reduction of cytopathogenicity by FFV.

INTRODUCTION

Foamy viruses (FVs) are classified in the genus *Spumavirus* of the family *Retroviridae* and can be isolated from many mammals including cats, cattle, humans and nonhuman primates (Lecellier and Saïb, 2000). FV infection is usually inapparent and there is no known association with clinical disease (Loh, 1993). Although the FV genomic DNA can be detected in various organs of infected animals (Hooks and Gibbs, 1975), genome transcripts are detected only in the oral mucosa (Falcone *et al.*, 1999). Therefore, the FV replication may be highly suppressed in most tissues *in vivo*. However, infectious FV can readily be recovered by inoculation of susceptible cell lines with various tissues, peripheral blood, or throat swabs from infected hosts (Shroyer *et al.*, 1978; von Laer *et al.*, 1996). The FV replication *in vitro* is accompanied by marked cytopathic effect (CPE) characterized by large syncytia formation and vacuolated cytoplasm (Hooks and Gibbs, 1975). Some of the cells that survive from acute infection can be passed as a stable line with very weak CPEs (Miyazawa *et al.*, 1995; Yu *et al.*, 1996-c). An understanding of the regulation of the persistent infection in the cells is important for understanding how a long-term, persistent infection can be maintained without any disease manifestations *in vivo*.

Like other FVs, feline foamy virus (FFV) uses a unique replication system, which is characterized by efficient utilization of an internal promoter (IP) to direct expression of regulatory and accessory genes, *bel*-1, 2 and *bet*,

located between *env* and 3' long terminal repeat (LTR) (Löchelt *et al.*, 1993-a; Löchelt *et al.*, 1994; Mergia *et al.*, 1994). The IP is initially activated by Bel-1 protein, also called Tas, for transactivation of FFV genes. Subsequent transactivation of the 5' LTR promoter by the protein induces synthesis of structural virus genes (Keller *et al.*, 1991; Löchelt *et al.*, 1995; Rethwilm *et al.*, 1991; Zou and Luciw, 1996). Other accessory gene products, Bet and Bel-2, are also expressed at the same time as the Bel-1 in the infected cell cultures (Bodem *et al.*, 1998). However, the functions of Bet and Bel-2 proteins are unknown. Recently, it has been reported that reduced infectivity of FFV occurs in the absence of the functional *bel*-2 gene (Alke *et al.*, 2001). This observation suggests that either the *bel*-2 or related accessory genes may play a key role for FFV replication.

In this study, the author describes transcriptional patterns of viral mRNAs in the acute and persistent FFV infections.

MATERIALS AND METHODS

Cells and viruses.

Methods for cultivation of Crandell feline kidney (CRFK) cells, propagation of Coleman strain of FFV, and activation of virus replication from FFV-persistently infected cell cultures with a histone deacetylase inhibitor (trichostatin A; TA) were as described previously (Chapter 1, Miyazawa *et al.*, 1995). Infected cells were harvested 4 days post infection (p.i.), washed with phosphate buffered saline (PBS) and used for isolation of RNA or analysis of proteins by SDS-PAGE.

Extraction of total RNA and northern blot analysis.

Total cellular RNAs were isolated 4 days p.i. by ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions, separated on 1.2% agarose-formaldehyde gel (10 μg per lane) and blotted to a nylon membrane (Biodyne B, Pall BioSupport, NY, USA). FFV specific DNA probes of *bel*-1 and *bel*-2 regions were prepared from an FFV infectious clone, pSKY3.0 (Chapter 3) by polymerase chain reaction (PCR) amplification with *pfu* DNA polymerase (Life Technologies, Inc., Rockville, MD, USA). The PCR products were gel purified with the QIAEX II gel extraction kit (Qiagen, Hilden, Germany), labeled with horse radish peroxidase (HRP) (ECL Direct Nucleic Acid Labelling System, Amersharm Pharmacia Biotech, NJ, USA), and hybridized to the blotted RNA. Hybridization was detected by enhanced

chemiluminescence (ECL Detection Reagent, Amersham). Two sets of primer pairs used for PCR were as follows; *bel*-1F, 5'atggcttcaaaatacccgga3' (nt. 9228-nt. 9247) and *bel*-1R, 5'acggactcagaattggaagg3' (nt. 9708- nt. 9727); *bel*-2F, 5'taaaaccgacccggattgtg3' (nt.9954-nt. 9973) and *bel*-2R, 5'atttatatggcacaataattc3' (nt. 10429-nt. 10449).

Preparation of Bel-1- and Bel-2–specific antiserum and western blot analysis.

Each of the *bel*-1 and *bel*-2 DNA fragment was amplified by PCR with *pfu* DNA polymerase, inserted into the pGEX-4T vector (Amersham) by blunt/blunt ligation to create an in-frame fusion to glutathione S-transferase (GST). The Bel-1- and Bel-2-GST fusion protein was individually expressed in XL-1 Blue strain of *E.coli*, separated by 10% SDS-PAGE, excised the appropriate protein band after staining by coomasie-brilliant-blue, homogenized the gels and eluted into PBS by dissemination method. Proteins for immunization were emulsified in Freund's complete adjuvant (Rockland, Inc., PA, USA) for the initial injection or Freund's incomplete adjuvant for booster injections. The ddY mice were immunized with the fusion proteins twice at intervals of 3 weeks by intraperitoneal route. Sera were harvested 7 days after booster injection and analyzed for specific antibody by western blots. Western blots were done using FFV- or mock-infected CRFK cells that were harvested on 4 days p.i., mixed with SDS-sample-loading-buffer, separated by 12% SDS-PAGE, and transferred to

nylon membranes (Hybond-C extra, Amersham). Transferred proteins were reacted with FFV-specific sera and HRP-conjugated second antibody, and detected by histochemical staining with diaminobentidine.

RESULTS

Transcriptional patterns of FFV in acute and persistent infections.

The author compared the transcription patterns of FFV in acute and persistent infections of FFV in CRFK cells. The transcriptional map (Fig. 1A) of the FFV has been reported by Bodem *et al.* (1998). The transcripts of 10.5, 8.9 and 7.6, and 5.6 kb correspond to mRNAs for structural genes *gag*, *pol* and *env,* respectively. The 10.5 kb mRNA is also the genomic RNA which is incorporated into virion. The transcripts of 2.6, 2.2 and 2.0 kb correspond to mRNAs for regulatory genes, *bel*-1, *bet* and *bel*-2, respectively. The 10.5, 8.9 and 5.6 kb transcripts are regulated by the promoter in the 5' long terminal repeat (LTR). On the other hand, the 2.6, 2.2 and 2.0 kb transcripts are regulated by the internal promoter (Bodem *et al.*, 1998). The 7.6 kb of transcript, which presumably encode the mRNA for *pol* gene, has not been reported. Significant differences were observed in the transcripts encoding the regulatory genes (Fig. 1B). Three transcripts (2.6, 2.2 and 2.0 kb) were detected only in the acute infection. In contrast, only 2.6 kb transcript (for *bel*-1) was faintly detected in the persistent infection. These findings indicate that the IP is active in the acute infection, but suppressed in the persistent infection.

The transcriptional patterns for structural genes were also different between the acute and persistent infections. Although the 5.6 kb transcript (for *env*) was weakly detected in the acute infection, the mRNA species was

strongly detected in the persistent infection. The 8.9 kb transcript (for *pol*) is detected in the acute infection, but not in the persistent infection, and the transcript was replaced by the 7.6 kb transcript.

Transcriptional pattern of FFV in TA-reactivation

Previously the author reported that FFV can be reactivated from the persistently infection by the treatment of a histone deacetylase inhibitor TA. The activation of the promoter in LTR was accompanied by the enhancement of the viral production (Chapter 1). The transcriptional pattern in the TA reactivation was similar to that in the persistent infection (Fig.1B). The 2.6 kb transcript (for *bel*-1) was weakly detected in the TA reactivation.

Bet/Bel-2 expression levels are decreased during persistent FFV infection.

Western blot analysis was conducted to compare the gene expression in the acute and persistent infections and TA-reactivation (Fig. 2). The 52 kDa and 48 kDa of Gag proteins were detected by the serum from an FFV-infected cat in the acute, persistent infections and TA-reactivation. The 35 kDa protein band is also characteristically detected both the persistent infection and TA-reactivation, but the author could not identify this protein band. By this cat serum, the author could not detect the regulatory proteins (Fig. 2C). Therefore, we prepared polyclonal antibodies by immunization of mice with Bel-1 and Bel-2 peptides as described in Materials and Methods.

The prepared antibodies were clearly detected the Bel-1 and Bel-2 peptides expressed in *E. coli* (Fig. 2B). In the acute infection, 44 kDa of Bet and 23 kDa of Bel-1 proteins and the Bet and 36 kDa of Bel-1 proteins were detected by the Bel-1 and Bel-2 specific antibodies, respectively (Fig. 2C). However, in the persistent infection and TA-reactivation, the Bet and Bel-2 proteins could not be detected and the Bel-1 protein was faintly detected.

DISCUSSION

In this chapter, the author examined the patterns of mRNA transcription in FFV infected cells, which may be influenced by the promoter activities of IP and LTR, during acute and persistent infections.

The transcriptional patterns of human foamy virus (HFV) in the acute and persistent infections was reported by Meiering *et al.* (2001). In their study, strong transcriptional activities of both LTR promoter and IP were observed in the acute infection, whereas strong transcriptional activity of IP and very low activity of the LTR promoter were detected in the persistent infection (Meiering *et al.*, 2001). However, they used different cell lines in the acute and persistent infections, therefore it is still uncertain whether the difference of HFV transcription represents the difference between the acute and persistent infections. Previously, Miyazawa *et al.* (1995) established the persistent infection as well as acute infection in the CRFK cells. To know the mechanism of FFV persistence *in vivo*, the author compared the patterns of the transcription between the acute and persistent infections in the cells.

In the acute infection of FFV, viral transcriptions from LTR promoter as well as IP were very active. Although the transcription from LTR promoter was maintained in the persistent infection, down-regulation of IP leading to the very low transcription of *bel*-1 mRNA was observed. Western Blot analysis also supported this result. This alteration of patterns of

mRNA transcription presumably owes the difference between the acute and persistent infections. LTR promoter is activated by IP transcription followed by Tas expression (Keller *et al.*, 1991; Löchelt *et al.*, 1995; Rethwilm *et al.*, 1991; Zou and Luciw, 1996). However, the LTR promoter activity may be maintained by small amounts of Tas expressed in the persistent infection. Splicing patterns of mRNAs for structural proteins were also changed. Relative ratio of genomic RNA transcripts and *env* mRNA decreased in the persistent infection when compared with that in the acute infection. The *genomic* RNA is responsible for Gag expression, therefore, it might be possible that the persistent infection is maintained by altering the expression ratio of each viral component.

Interestingly, TA treatment did not activate the IP, but slightly activates the LTR promoter. These findings are consistent with the results of Western Blot analysis. The author has described that TA induces the FFV production from the CRFK cells persistently infected FFV (Chapter 1). However, the transcription pattern in the TA reactivated cells is not the same as the acute infection. Thus, TA is likely to activate the LTR promoter which is integrated in host genome without activation of IP.

Both Bet and Bel-2 proteins were highly expressed during the acute infection, while neither the Bet nor the Bel-2 was detected in the persistent infection. The expected transcripts were also detected by Northern Blot analysis. Although functions of Bet and Bel-2 are unknown, these observations may indicate that the Bet and Bel-2 play an important role for

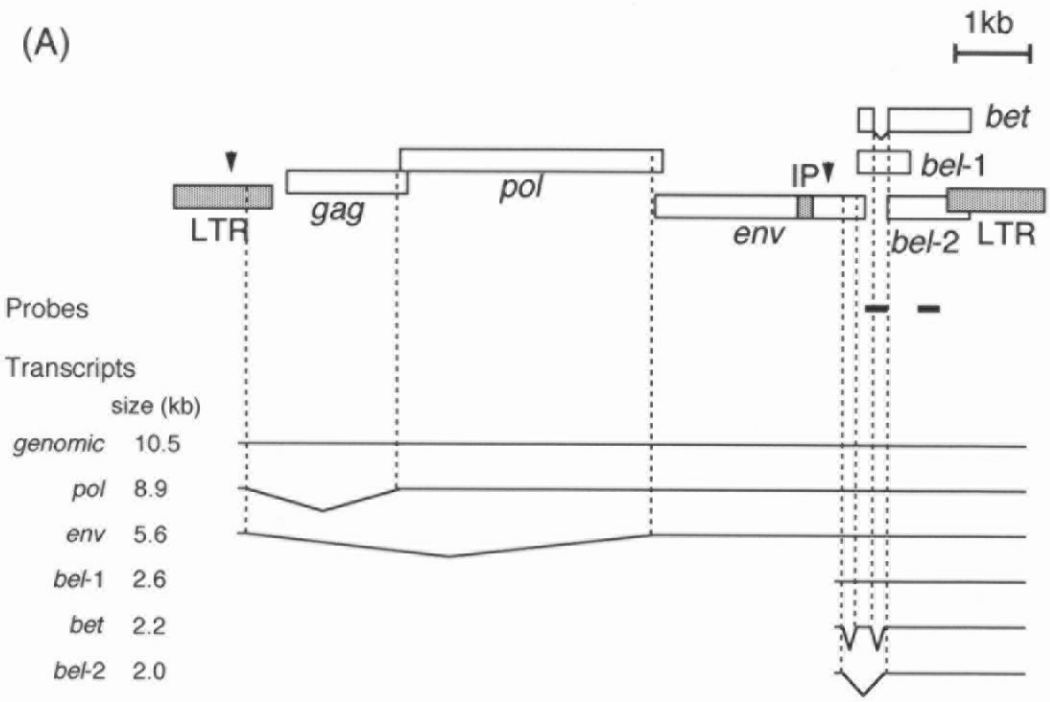
the acute infection. The titers of *bel*-2-gene-defective FFV has been reported to be 1000-fold less in culture supernatant than wild-type virus (Alke *et al*, 2001). Although *bel*-2 gene may not be essential for FFV replication, it is possible that *bel*-2 gene related proteins are required for budding or releasing of virus particles. Further analysis of the function of Bet and Bel-2 may insight into the persistent mechanism of FFV.

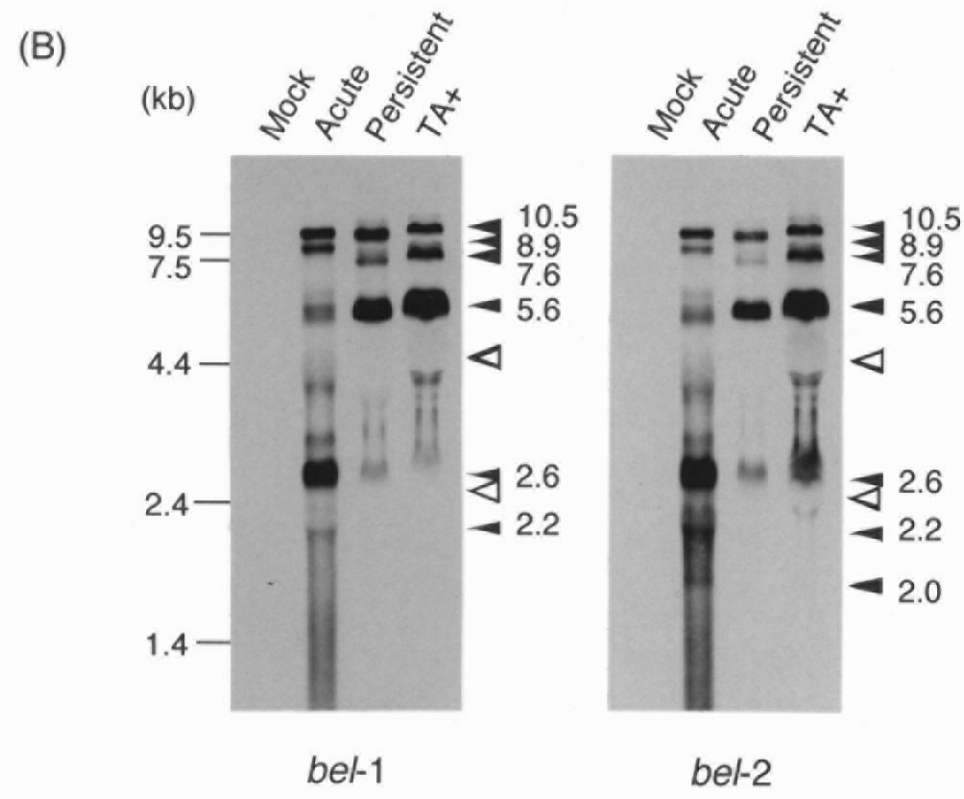
FIGURE LEGENDS

FIG. 1. Transcriptional analysis of FFV infected CRFK cells. (A) Schematic diagram of the FFV proviral genomic organization and transcripts identified by the RT-PCR and DNA sequences are depicted. Open and shaded boxes indicate the FFV genes and promoter sequences, respectively. The vertical arrow heads mark the position of the 5' LTR and the internal start sites of transcription. The positions of hybridization probes are designated in *bel*-1 and *bel*-2 introns, and marked by a thick line. The names of the transcripts which were detected in this experiment were quoted from the previous report (Bodem *et al.*, 1998). The *genomic*, *pol*, and *env* are derived from the 5' LTR promoter, while *bel*-1, *bet* and *bel*-2 are derived from IP. (B) Northern blot hybridization was performed for total RNAs from the CRFK cells mock-, acutely- or persistently-infected with FFV. Persistently infected cells were reactivated by TA treatment and also served for the northern blot analysis. The FFV specific DNA probes were labeled by horse radish peroxidase, hybridized to the RNA blot and detected by enhanced chemiluminescence. Solid arrowheads show the sizes of transcripts, and open arrowheads denote location of 28S and 18S rRNA. The positions and sizes (kb) of the RNA markers (Gibco BRL, Grand Island, NY) are indicated at the left-hand margin.

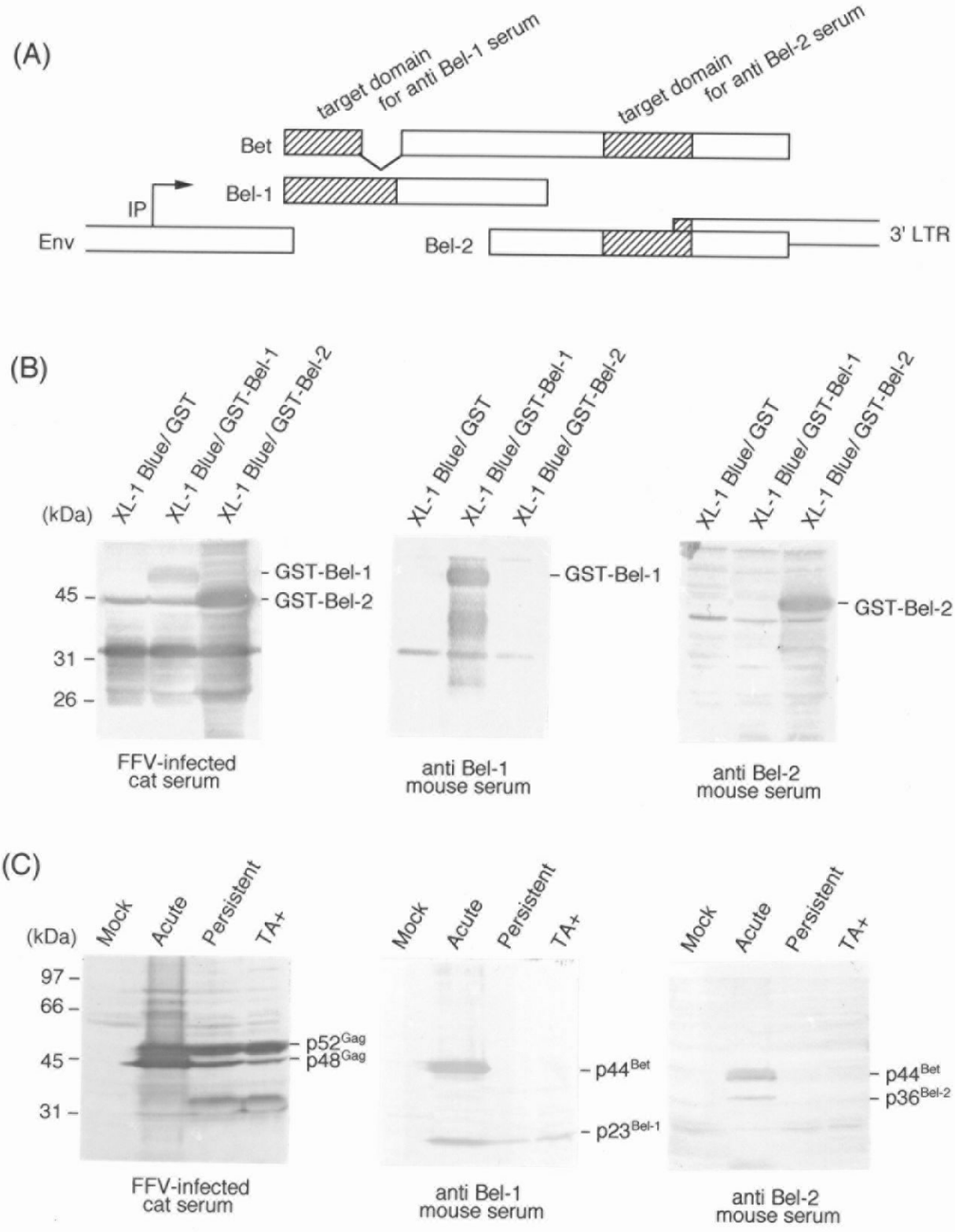
Fig. 2. Western blot analysis of FFV proteins. (A) 3' half region, between *env* and 3' LTR, of the FFV proviral genome is represented. Target domain to generate the anti Bel-1 and anti Bel-2 sera are depicted by shaded rectangles. (B) Either GST-tagged-Bel-1 or -Bel-2 protein was expressed in XL-1 Blue strain of *E. coli*, subjected to SDS-PAGE and transferred electrophoretically to membranes. The membranes were immunostained with FFV-infected cat serum, anti Bel-1 mouse serum and anti Bel-2 mouse serum. The molecular masses of marker proteins are shown to the left. (C) Different types of CRFK cell cultures infected or mock-infected with FFV were analyzed by immunoblotting with either the FFV polyclonal, Bet- or Bel-2-specific antiserum. Specifically detected proteins correspond to the FFV $p52^{Gag}$, $p48^{Gag}$, $p44^{Bet}$ and $p36^{Bel\text{-}2}$. The molecular masses of marker proteins are given at the left margin.

Chapter 2. Fig. 1.





Chapter 2. Fig. 2.



CHAPTER 3

Isolation and sequencing of infectious clones of feline foamy virus and human /feline foamy virus Env chimera

ABSTRACT

Full-length DNAs of the Coleman and S7801 strains (pSKY3.0, pSKY5.0) of infectious feline foamy viruses (FFVs) were cloned and sequenced. Parental viruses, designated SKY3.0 and SKY5.0, were secreted following transfection to Crandell feline kidney (CRFK) cells. Production of the rescued parental viruses was enhanced in the presence of trichostatin A (TA). Amino acid sequence homologies between FFV and human foamy virus (HFV) are extremely low for the envelope protein and capsid antigen, as predicted from the two clones. However, a chimeric FFV clone was constructed with the HFV Env substituted for the FFV Env. The chimeric virus (HFFV, SKY4.0) was able to infect and replicate in CRFK cells as well as in PBMC of cats *in vivo*. Consequently, the chimeric HFFV may be useful for the creation of FV vectors for gene transfer strategies.

INTRODUCTION

The genomic DNA of feline foamy virus (FFV) contains structural genes designated *gag*, *pol* and *env*, as well as the auxiliary gene *bel* (Helps and Harbour, 1997). These genes are located between two long-terminal repeats (LTRs). Although this type of genome organization is common for foamy viruses (FVs) from several species of mammals, there are remarkable dissimilarities between FVs and other retroviruses in their mode of replication (Yu *et al.*, 1996-a). For example, interactions between group-specific antigen (Gag) and envelope protein (Env) are essential for the assembly and budding of infectious FV particles (Pietschmann *et al.*, 1999), whereas the Gag protein alone is sufficient to allow budding in cells infected with retroviruses other than FVs (Linial, 1999). Reports indicate that it is difficult to produce high titer pseudotyped FV bearing glycoproteins from murine leukemia virus Env or vesicular stomatitis virus G proteins (Lindemann *et al.*, 1997; Hill *et al.*, 1999). These observations indicate that the interaction between FV Gag and Env of different species origin may be of interest when evaluating FVs as vector candidates for gene transfer. The author reports the isolation and sequencing of FFV clones SKY3.0 and SKY5.0. Furthermore, the author shows that a chimeric FFV clone (HFFV) bearing Env and a part of the trans-activator (Tas) from human foamy virus (HFV) can infect and replicate in HFV-susceptible cells.

MATERIALS AND METHODS

Viruses and cells

Coleman, S7801 and Sammy-1 strains of FFV were grown as described previously (Chapter 1). The HFV strain, HS007 (Adachi *et al.*, 1995), was a kind gift of Dr. A. Adachi, The University of Tokushima School of Medicine.

Construction of plasmids

Crandell feline kidney (CRFK) cells were infected with each of FFV strain, and total DNA was extracted using the Total DNA Extraction Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Preliminary experiments revealed that the extracted DNA contained a number of circular forms of unintegrated viral DNA, which contains single LTR. To construct the infectious clones, pSKY1.0 and pSKY2.0, the extracted DNA was amplified by polymerase chain reaction (PCR) using two primer pairs, 5'-agctgatgatccaagtgatgttgcttccc-3' (nucleotide (nt) 10239-10267 in the FUV genome; accession number Y08851) and 5'-cgactcatcctgagttgcatgttgacata-3' (nt 6395-6423), and 5'-ggaatggaatgctcacaaacaactacaga-3' (nt 6357-6385) and 5'-ctagggaccttaccttactgaggaaggat-3' (nt 1415-1443). Amplified DNAs were cloned into pCR2.1 (Invitrogen, Groningen, The Netherlands) to give two clones designated as 5' LTR-*gag-pol* (pSKY1.0) and *env-bel*-3' LTR (pSKY2.0).

To construct the full-length Coleman clone, pSKY3.0, the *Sal* I-*Spe* I double digested fragment of pSKY2.0 was inserted into pSKY1.0 which had been digested with the same enzymes. A similar procedure was used to construct the full-length S7801 clone, pSKY5.0. To investigate whether HFV Env and FFV Gag proteins can assemble together, an HFV Env (Tas)-chimeric clone was constructed. The infectious clone of HFV-N (pHS007) has been described previously (Adachi *et al.*, 1995). Nucleotide sequence representing the full-length *env* and a part of *bel*-1 of HFV-N (3229 bp) was amplified by PCR using primers 5'-aaatgaataaagcgcatgagg-3' (nt 7185-7205) and 5'-taaaacagtcagggtcagtatc-3' (nt 10444-10465). The amplified fragment was inserted into the *Sal* I and *Mro* I double digested pSKY3.0 after blunting the ends (HFFV, pSKY4.0; accession number AB052798) (Fig. 1A). The fragment representing the HFV *env* and a part of *bel*-1 was substituted for the equivalent region of FFV (aa 1-129 of HFV Tas, I was converted to T at aa 130, and fused with aa 131-209 of FFV Tas) (Fig. 1A).

Nucleotide sequence analysis

The DNA sequences of the FFV clones, pSKY3.0 (accession number AB052796) and pSKY5.0 (accession number AB052797) were determined using a DSQ-2000L DNA sequencer (Shimazu Co., Ltd., Tokyo, Japan).

Transfection of plasmids and production of infectious clones

FV plasmid DNA (20 μg/ml) was transfected into FFV or HFV

permissive cells (5 x10^6) using 5 μg of lipofectin (Gibco, Grand Island, NY) in the presence of 10^{-5} M of trichostatin A (TA), as described previously (Chapter 1). Cells (1 x 10^5) were infected with 0.1 ml of the cell-free supernatants, and cultured with 1 ml of the complete medium. The viral titer was measured daily for 7 days using a CPE assay with endpoints reported as $TCID_{50}$ (Ikeda *et al.*, 1997).

Expression of His-tagged Gag protein

The recombinant FFV *gag*-expressing plasmid pFFV/01 was constructed by inserting the blunted *Xho* I-*Sca* I fragment (nt 1525-3696) into the *Sal* I- digested and blunted pQE02 plasmid (Qiagen, Hilden, Germany). The His-tagged Gag protein was extracted and purified using Ni-NTA column chromatography (Qiagen).

Detection of viral specific DNA by PCR

Three pairs of primers, 5'-aggacctgaaagacatg-3' (nt 1732-1748) and 5'-ttgttgagatcgtccctg-3' (nt 2528-2545) for FFV *gag*, 5'-tatgtccctaggagagg-3' (nt 8172-8188) and 5'-aagcttgttagccgagg-3' (nt 8890-8906) for FFV *env* and 5'-tgaagaaaatcctcgacgcc-3' (nt 9416-9435) and 5'-ttgacgtttgcttcggcatg-3' (nt 9727-9746) for chimeric *bel*-1, were used for the first round PCR. Three additional pairs, 5'-aggacctgaaagacatg-3' (nt 1732-1748) and 5'-agcggctgtagatcttcc-3' (nt 2039-2056) for FFV *gag*, 5'-tatgtccctaggagagg-3' (nt 8172-8188) and 5'-ttcccacgcactagaag-3' (nt 8384-8400) for FFV *env* and 5'-

cccacaccagaggaaatgag-3' (nt 9537-9746) and 5'-ttgacgtttgcttcggcatg-3' (nt 9727-9746) for chimeric *bel*-1, were used for the second round of PCR amplification.

RESULTS

Nucleotide sequence of infectious FFV clones

The Coleman and S7801 clones were 11,694 and 11,660 bp in length. Comparisons of the putative Gag, Pol, Env, Bel-1 and Bel-2 amino acid (aa) sequences between either SKY3.0 or SKY5.0 and FUV (Winkler *et al.*, 1997) gave homology estimates of 95.9, 96.9, 82.4, 89.8 and 94.2%, and 96.9, 95.4, 83.3, 94.3 and 93.6%, respectively. Gag, Pol, Env, Bel-1 and Bel-2 aa sequence homologies between either SKY3.0 or SKY5.0 and HFV (Löchelt *et al.*, 1991) were 34.7, 60.1, 42.3, 17.6 and 31.8%, and 34.4, 59.7, 43.0, 46.2 and 36.4%, respectively.

In vitro infectivity of infectious FFV clones

CRFK cells infected with either the Coleman strain of FFV or SKY3.0 showed cytocidal effects with the formation of large syncytia (Fig. 1B-b, c). HFV-N as well as SKY4.0 also produced marked CPE in BHK-21 cells (Fig. 1B-e, f). A non-cytopathic, persistent infection was established following 4 passages of SKY3.0 or SKY4.0 infected cells with uninfected cells. SKY3.0 and SKY4.0 DNAs were detected by PCR in the persistently infected cells, as well as in acutely infected CRFK and BHK-21 cells (data not shown). These data indicate that the cloned FFV and HFFV DNAs can persist in the cells after repeated passages. Transfection of pCR2.1 as a negative control did not produce CPE in CRFK or BHK-21 (Fig. 1B-a, d). Cell-free

supernatants from pSKY3.0, pSKY4.0 and pSKY5.0 transfected CRFK cells yielded approximately 10^2 $TCID_{50}$ after 30 h. After infection with supernatants from transfected cells, titers of SKY3.0 and 5.0 parental viruses on day 3 were $>10^6$ and $>10^3$ $TCID_{50}$/ml, respectively (Fig. 1C). Titers of SKY1.0 plus SKY2.0 viruses were lower (approximately 10^3 on day 4) than when full-length recombinant clones were used. The titer of SKY4.0 recombinant virus (HFFV) was approximately 10^5 $TCID_{50}$/ml on day 3 (Fig. 1C).

The cytopathic effects of FFV, HFFV and HFV-N were examined on CRFK, BHK-21, Gin-1, HeLa, HL60 and PC12 cells (Table 1). HFFV derived from pSKY4.0 induced syncytia formation in HFV- susceptible BHK-21 cells, but not in the Gin-1, HeLa or HL60 human, or the CRFK feline, cell lines (Table 1). FFV and HFV derived from pSKY3.0 and pHFV-N caused the expected syncytia formation in CRFK and BHK-21 cells. CRFK, BHK-21, Gin-1, HeLa and HL60 cells contained FFV-, HFFV- and HFV-specific *gag* DNA that was detectable by PCR (Table 1). These results indicate that the pattern of CPE produced by the chimeric viruses in various mammalian cell lines is consistent with the species of origin of the *env* gene, and that FV receptors are all similarly distributed on different mammalian cell lines.

In vivo infectivity of infectious FFV clones

To investigate the infectivity of the FFV (SKY3.0) and HFFV (SKY4.0) molecular clones *in vivo*, FVs were injected into four SPF male cats

(cats A and B; SKY3.0, cats C and D; SKY4.0). Cats were infected by the i.p. route with $10^{4.5}$ $TCID_{50}$ of FFV from supernatants of infected cells and sera were obtained at 30 days post-infection. Antibodies against FFV and HFFV were detected in the FV infected cats by immunoblotting (Otake *et al.*, 1994). Serum from SKY3.0-infected cat B was used to probe lysates of Coleman strain or SKY3.0-infected CRFK cells and SKY4.0- or HFV-N-infected BHK-21 cells (Fig. 2A). Bands of the 48 and 52 kDa Gag proteins were observed in infected cells, but not in mock-infected or HFV-N infected cells (Fig. 2A). Serum from the mock-infected cat #1 did not react with the Gag proteins (Fig. 2A). Similar results were obtained using sera from cat A, C and D (data not shown). His-tagged Gag protein was expressed in *E. coli* and also used as a target antigen for immunoblotting with the cat sera. Sera containing anti-Gag polyclonal antibody were obtained from rabbits hyperimmunized with purified His-tagged Gag. Sera from cats B and C reacted with the 52.5 kDa recombinant Gag protein (Fig. 2B), while pre-immune sera were non-reactive (data not shown). Ni-NTA alkaline-phosphatase conjugates and anti-Gag rabbit serum reacted with the 52.5 kDa bands (Fig. 2B). Serum from mock-infected cat #1 did not react with the Gag protein bands. Both pre-infection and infected sera from cats A and D, infected with SKY3.0 and SKY4.0, respectively, gave results comparable to those obtained with sera from cats B and C (data not shown). To investigate whether viral genomes were present in the peripheral blood mononuclear cell (PBMC) of infected cats, total DNA was extracted from PBMC at 30 days post-infection and analysed by nested-

PCR. FFV *gag* specific DNA was detected in SKY3.0- and SKY4.0-infected cats (Fig. 2C). FFV *env*- and chimeric *bel*-1-specific DNAs were detected only in cats infected with SKY3.0 and SKY4.0, respectively (Fig. 2C). The PBMCs from the negative control, mock-infected cat #1, yielded no bands, while the pSKY3.0 and pSKY4.0 positive control gave the expected FV specific bands (Fig. 2C). Therefore, these results suggest that SKY3.0 and SKY4.0 are replication-competent *in vivo*, and the viral DNA is harbored in the PBMC for at least 30 days after infection.

DISCUSSION

In this chapter, the author suggests the feasibility of using chimeric FFV genomes to produce replication-competent FFV particles. Low titers of cell-free, secreted FV are a practical disadvantage for exploitation of FV clones as vectors (Zemba *et al.*, 2000). The use of TA as a supplement in the transfection procedure (Chapter 1), along with the use of CRFK cells containing β-*gal* reporter plasmids as a sensitive detector system (Yu and Linial, 1993) gives extracellular titers of approximately 10^8 FFU/ml. The titers may be greater than those obtained previously for HFV by Yu and Linial (1993). The functional domains of HFV Tas, promoter-targeting domain, nuclear localization signal and transcription activation domain, have been reported to located within the central and carboxyl-terminal region of the Tas protein (He *et al.*, 1996). The chimeric Tas of HFFV contains aa 1-129 from the amino-terminal region of HFV Tas and aa 131-209 from the carboxyl-terminal region of FFV Tas. Although aa sequence homology was low between HFV and FFV Tas, the chimeric *tas* gene was functional in generating a viral protein which is able to activate the FFV promoter. These results indicate that the major functional domains of the chimeric Tas are probably localized in central to carboxyl terminal region. Thus, HFFV is likely to be a highly useful tool for a DNA delivery system. In previous studies, attempts to pseudotype HFV using Env from other retroviruses did not result in secretion of particles (Lindemann *et al.*, 1997; Pietschmann *et al.*,

1999). However, the author found that the HFFV chimera can produce progeny viruses. Thus, it is suggested that HFV Env can interact with the FFV Gag protein to give a productive replication cycle. Although several cell lines are susceptible to HFV *in vitro* (Hooks and Gibbs, 1975), these results indicate that HFV- (Russell *et al.*, 1996) as well as FFV-based vectors may be suitable for gene transfer.

FIGURE REGENDS

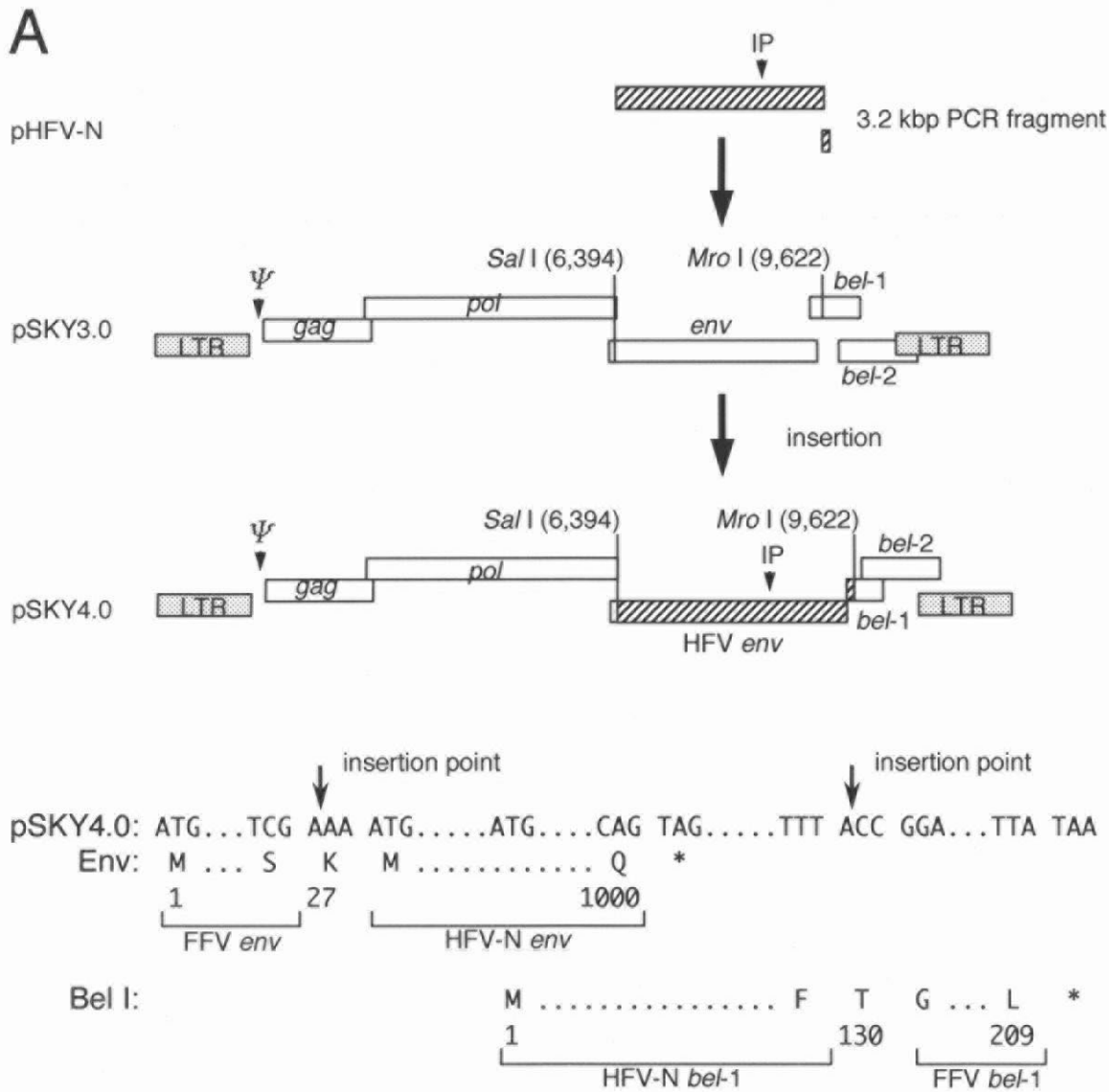
Fig. 1. **A.** Insertion of the HFV *env* and a part of the *bel*-1 genes into FFV genome. The full-length FFV Coleman clone (pSKY3.0) was constructed from clones pSKY1.0 (5'-half) and pSKY2.0 (3'-half). The entire *env* region from an infectious HFV-N clone was amplified by PCR. The chimeric FFV with HFV *env* plasmid (pSKY4.0) was constructed by inserting an approximately 3.2 Kbp fragment (*env* and a part of *bel*-1) from pHFV-N into the *Sal* I (nt 6,394)-*Mro* I (nt 9,622) blunted site of pSKY3.0. The predicted initiation and termination sites of the *env* and *bel*-1 ORFs, and both sites of the insert are depicted. Exact insertion points are represented by arrowheads in the nucleic acid sequences. Amino acid residues and numbers are also shown. IP; internal promoter. **B.** Morphological alteration of cells infected with chimeric virus. Typical syncytia were formed in CRFK cells infected with FFV Coleman strain (b) and SKY3.0 (c), and BHK-21 cells infected with HFV-N (e) and SKY4.0 (f). The respective mock-infected cells are shown in panels (a) and (d). The plaque morphologies are observed at ×200 magnification after staining with May Grünwald-Giemsa. **C.** Replication kinetics of parental viruses from infectious DNA clones. The FFV infectious clones, pSKY1.0 plus pSKY2.0, pSKY3.0 and pSKY5.0 were transfected into CRFK cells and the HFFV chimeric clone,

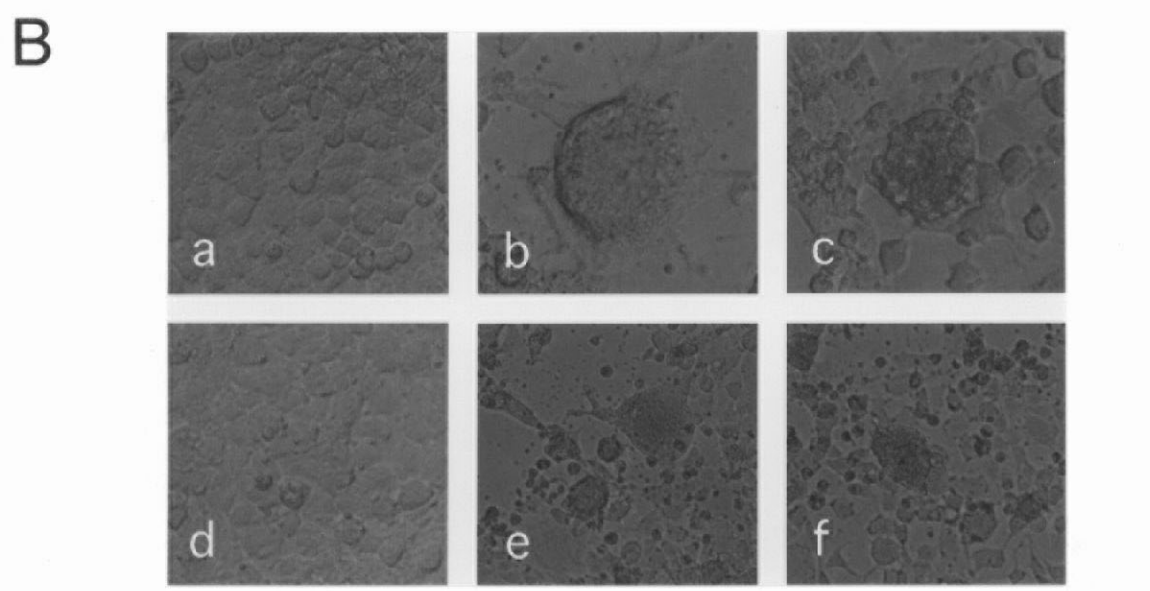
pSKY4.0, was also transfected into BHK-21 cells. At 30 h post transfection, the appropriate parental viruses were secreted into the culture supernatant and injected to the cells in the presence of TA. The culture media from the infected cells was harvested at intervals of 24 h and viral titers were determined by $TCID_{50}$/ml. SKY1.0 + SKY2.0 (■), SKY3.0 (□) and SKY5.0 (●) and SKY4.0 (▲)

Fig. 2. **A.** Expression of Gag protein from infectious chimeric clones. The lysates of CRFK, BHK-21, Coleman-strain infected CRFK, SKY3.0 infected CRFK, SKY4.0 (HFFV) infected BHK-21 and HFV-N infected BHK-21 cells were analyzed by SDS-PAGE. Immunoblotting was performed with SKY3.0 infected cat B serum and mock-infected cat #1 serum. **B.** Expression of Gag protein in *E. coli.* Six histidine (X6His)-tagged Gag protein of FFV Coleman-strain was expressed in *E. coli.* The X6His-Gag protein was purified by nitrilotriacetic acid (NTA) column chromatography, subjected to SDS-PAGE and transferred electrophoretically to membranes. The membranes were immunostained with mock-infected cat #1 serum, alkaline phosphatase labeled Ni-NTA, the X6His-tagged Gag protein immunized rabbit serum; SKY3.0 infected cat B serum and SKY4.0 infected cat C serum. The molecular mass of the expressed protein is shown to the left. **C.** Infectivity of the chimeric virus clone *in vivo.* In order to detect FV-

specific DNA from infected cats, total DNA was isolated from PBMC of SKY3.0 infected cat B and SKY4.0 infected cat C at 30 days post-infection. Three sets of primers were designated for the FFV *gag*, *env* and HFV *bel*-1 plus FFV *bel*-1 regions and nested PCR was performed. Mock-infected cat #1 was the negative control and pSKY3.0 or pSKY4.0 DNAs were positive controls. For FFV-specific *gag* and *env* sequences, 230 bp and 325 bp were amplified, respectively. For the chimeric *bel*-1 sequence, 209 bp were amplified.

Chapter 3. Fig. 1.





Chapter 3. Fig. 1 continued

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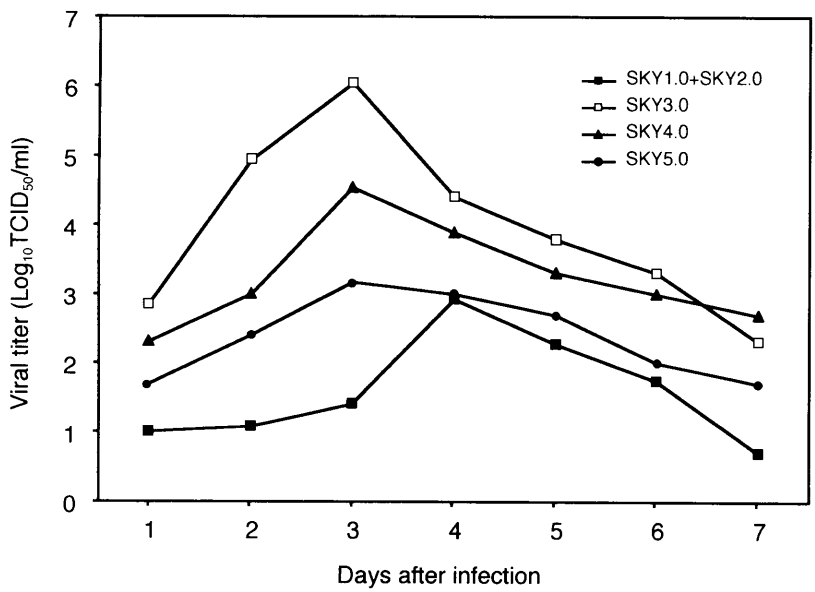
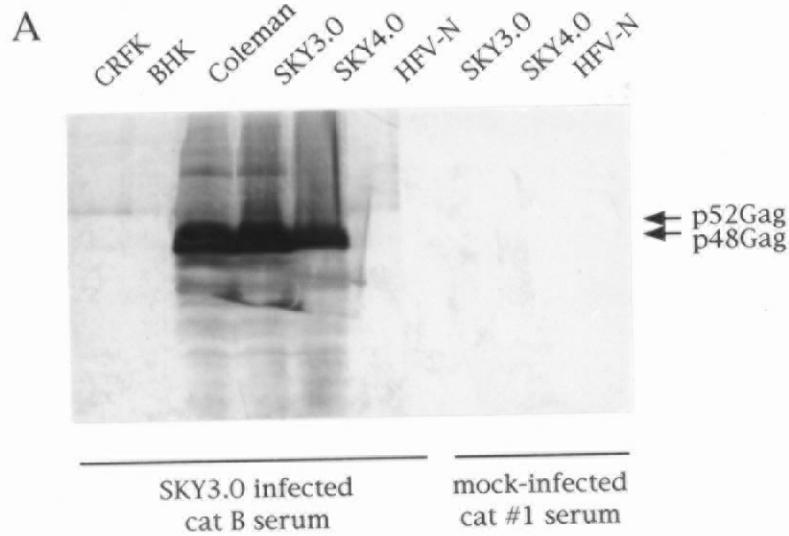
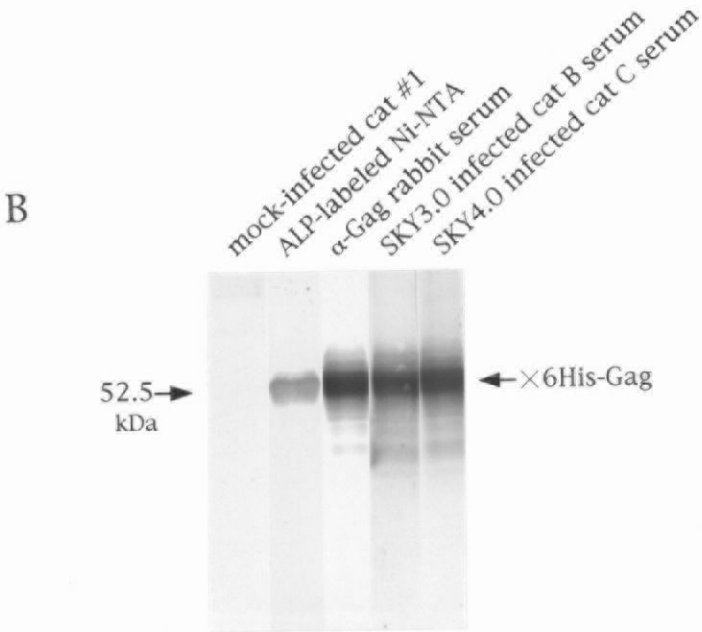


Chapter 3. Fig. 2.





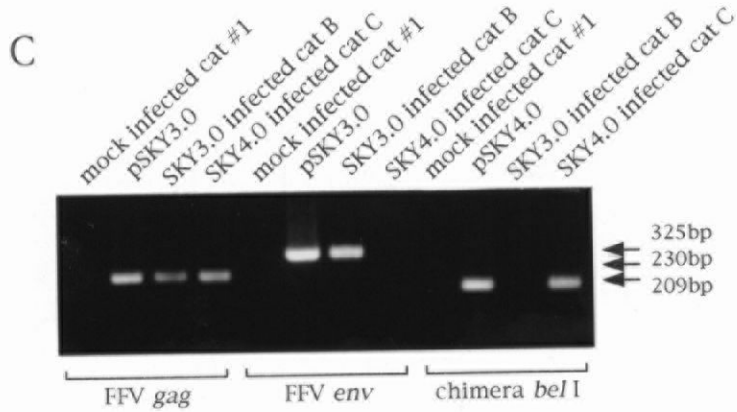


Table 1. Susceptibity of cell lines to infectious foamy virus clones.

Cells were infected with culture supernatant of trasfection with infectious virus clones. After three days post-infection, cells were scored visually according to the proportion of cells which formed syncytia. The viral DNA was extracted from the cells and *gag* fragment was amplified by PCR. Detection of PCR products was performed with agarose gel electrophoresis. As a positive control, β-actin primers were used.

		Syncytia formation *			PCR **		
Cell line	Species/tissue	FFV	HFFV	HFV-N	FFV	HFFV	HFV-N
CRFK	Feline kidney	+++	-	-	+	+	+
BHK	Hamster kidney	-	+++	+++	+	+	+
Gin-1	Human gingival fibroblast	-	-	-	+	+	+
HeLa	Human epithelium	-	-	-	+	+	+
HL60	Human myelomonocyte	-	-	-	+	+	+
PC12	Rat adrenal medulla pheochromocytoma	-	-	-	±	±	±

*; +++, >30% positive ; -, negative

**:, +strong positive; ±, weakly positive

CONCLUSION

In Chapter 1, the author described that persistent CRFK cell culture infected with FFV can be activated by treatment of histone deacetylase inhibitor, Trichostatin A (TA). Viral production and FFV-specific CPE are increased but morphology of the viral particles does not alter by the TA treatment. TA enhances the transcription from LTR promoter of Coleman strain, but is not effective for the LTR promoter of S7801 or Sammy-1 strain. The entire LTR sequence is conserved in the Coleman strain as compared to the prototype FUV strain. However, both S7801 and Sammy-1 strains contain a partial deletion in the U3 region of their LTRs. Presence of the deletions may correlate with the inability of TA for enhancement of FFV replication. Other HAT or methylation reagents have less reactivity to FFV LTR. Therefore, the existence of complex mechanisms in FFV replication may be implicated. In this chapter, the author suggests that TA is a useful reagent to study the replication mechanisms of FFV.

In Chapter 2, the author described the difference of the gene expression between acute and persistent infection. IP products are efficiently expressed in acutely infected cells, but faintly expressed in persistently infected cells. Therefore, IP is characteristically activated during acutely infected cells. Genomic RNA transcription as well as Gag protein expression is similarly occurred during both types of viral replication, indicating that LTR activity is maintained. The consequence of the TA treatment does not convert the persistent replication to the acute one, but

likely to directly activate the LTR promoter.

In Chapter 3, the author described the generation and sequence analysis of infectious FFV molecular clones as well as hybrid FFV plus HFV clone. Some biological features of the recombinant viruses, such as cellular tropism and *in vivo* infectivity were studied. Since Env is essential for budding from FFV infected cells, Gag-Env interaction is absolutely required for the FFV infectious clones. Both of the FFV and HFFV molecular clones produce mature infectious viral particles *in vitro*, indicating that the FFV Env as well as HFV Env effectively interacts with FFV Gag. The HFFV genome contains full length of *env* and a part of *tas* genes of HFV in the FFV backbone. The HFFV is replication-competent not only *in vitro* but also *in vivo*. Therefore, the chimeric *tas* gene is functional in generating a protein which can activate the FFV promoter. Deduced from the low amino acid sequence homology between HFV Tas and FFV Tas, the major functional domains of the FFV Tas may be localized in central to carboxyl terminus of the FFV Tas sequence.

In this thesis, the author succeeded in construction of FFV and HFFV molecular clones. FFV does not manifest any clinical pathogenicity and proviruses can be stably integrated into host cells including non-dividing cells such as nerve cells or peripheral lymph cells. Therefore, although further studies are required, the FFV as well as HFFV molecular clone might

be helpful for exploitation of gene transfer vector intended not only for animal but also for human gene therapy.

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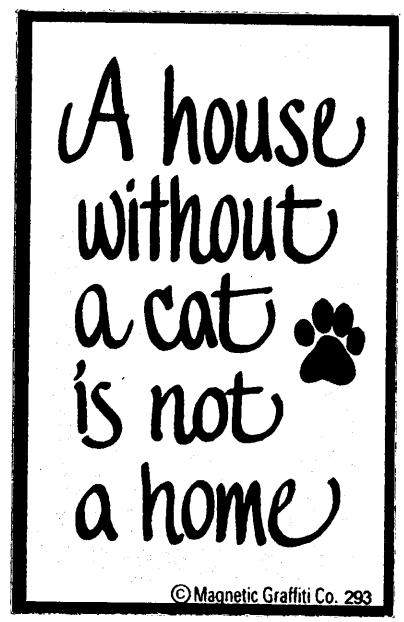
A house without a cat is not a home
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