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Roles of the Auxiliary Genes and AP-1 Binding Site in the Long Terminal Repeat of Feline Immunodeficiency Virus in the Early Stage of Infection in Cats

YASUO INOSHIMA,[1] MARIKO KOHMOTO,[1] YASUHIRO IKEDA,[1] HIROSHI YAMADA,[1] YASUSHI KAWAGUCHI,[1] KEIZO TOMONAGA,[1] TAKAYUKI MIYAZAWA,[1] CHIEKO KAI,[1] TAKASHI UMEMURA,[2] AND TAKESHI MIKAMI[1]*

Department of Veterinary Microbiology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113,[1] and Department of Veterinary Pathology, Faculty of Agriculture, Tottori University, Koyama, Tottori 680,[2] Japan

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To examine the roles of auxiliary genes and the AP-1 binding site in the long terminal repeat of feline immunodeficiency virus (FIV) in vivo, three mutant viruses, which are defective in the *vif* gene (Δ*vif*), ORF-A gene (ΔORF-A), and AP-1 binding site (ΔAP-1), and wild-type virus as a positive control were separately inoculated into three specific-pathogen-free cats. These cats were assessed by measuring the number of proviral DNA copies in peripheral blood mononuclear cells (PBMCs), the CD4/CD8 ratio and antibody responses to FIV for 16 weeks and then examining histological changes at necropsy. Although viral DNAs were detected in PBMCs from all 12 cats to various degrees until 16 weeks postinoculation, no virus was recovered from PBMCs of cats infected with Δ*vif* virus during the observation period. However, a very weak antibody response was induced in one cat infected with the Δ*vif* virus. In contrast, despite the successful recovery of virus from both groups of cats infected with ΔORF-A and ΔAP-1 virus, antibody responses and decrease in the CD4/CD8 ratio in the groups were milder than those in cats infected with wild-type virus. Furthermore, the numbers of proviral DNA copies in PBMCs from the two groups were not able to reach the level in cats infected with wild-type virus during the observation period. From these results, we conclude that these mutant viruses are still infectious for cats but failed in efficient viral replication and suggest that these auxiliary genes and enhancer element are important or essential to full viral replication kinetics and presumably to full pathogenicity during the early stage of infection in vivo.

Feline immunodeficiency virus (FIV) belongs to the *Lentivirus* genus of the retrovirus family, which includes human immunodeficiency viruses (HIVs), simian immunodeficiency viruses (SIVs), and ungulate lentiviruses (35). FIV is the etiological agent of immunodeficiency-like syndrome in cats (37) and is now known to occur worldwide (6). FIV genome contains at least three auxiliary genes, *vif*, ORF-A, and *rev* genes, in addition to three genes encoding the structural and enzymatic proteins, *gag*, *pol*, and *env*, that are in common with all retroviruses (46). Although the homologies of nucleotide and amino acid sequences of the auxiliary genes among the different lentiviruses show significant differences, it is believed that their functions are widely conserved in their natural host, and the genes play important roles in the viral life cycle (4).

In addition to the auxiliary genes, enhancer elements in the U3 region of the long terminal repeat (LTR), such as AP-1, AP-4, C/EBP, and ATF binding sites, are found in the FIV genome (10, 34). It is known that enhancer elements in the LTR of retroviruses are also considered to be important for virus expression, because the elements influence virus transcriptional activity directly in association with a variety of cellular transcription factors in vitro (4, 5, 9).

Previously, we and others have analyzed the functions of the *vif* and ORF-A genes and the AP-1 binding site of FIV in vitro and obtained the following information. (i) By deletion of the *vif* gene, the mutant produced viral reverse transcriptase (RT) at a normal level upon transfection to Crandell feline kidney (CRFK) cells, but cell-free virus prepared from the transfected cells did not infect a T-lymphoblastoid cell line (MYA-1 cells) and peripheral blood mononuclear cells (PBMCs). However, when MYA-1 cells were cocultured with transfected CRFK cells, the production of progeny virus was observed (48). Moreover, the FIV *vif* phenotype in vitro is dependent on host cell types from which the virus is derived (40, 48). (ii) The ORF-A-defective viruses, which had a deletion or a stop codon in the ORF-A gene, replicated with severely delayed kinetics compared with those of the wild-type virus in MYA-1 cells and PBMCs (38, 47). The phenotype of ORF-A-defective virus was also dependent on host cell types (50). Moreover, the ORF-A gene seemed to have only a low *trans*-activator activity, if any (33, 45, 47, 50). (iii) By deletion of the AP-1 binding site in the LTR, promoter activity decreased in various cells when examined by the chloramphenicol acetyltransferase assay (23, 33, 44, 45). However, the replication rate and the cytopathogenic activity of the AP-1 binding site-deleted mutant virus were almost the same as those of the wild-type virus in feline T-lymphoblastoid cell lines (MYA-1 and FeL-039 cells) and PBMCs (32, 33). On the basis of these results of in vitro experiments, here we examined the effects of deletions of the *vif* and ORF-A genes and the AP-1 binding site on viral load, immunological responses, and histological changes in cats and discussed the roles of these auxiliary genes and the site in the host.

* Corresponding author. Mailing address: Department of Veterinary Microbiology, Faculty of Agriculture, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan. Phone: 81-3-3812-2111. Fax: 81-3-5689-7346.

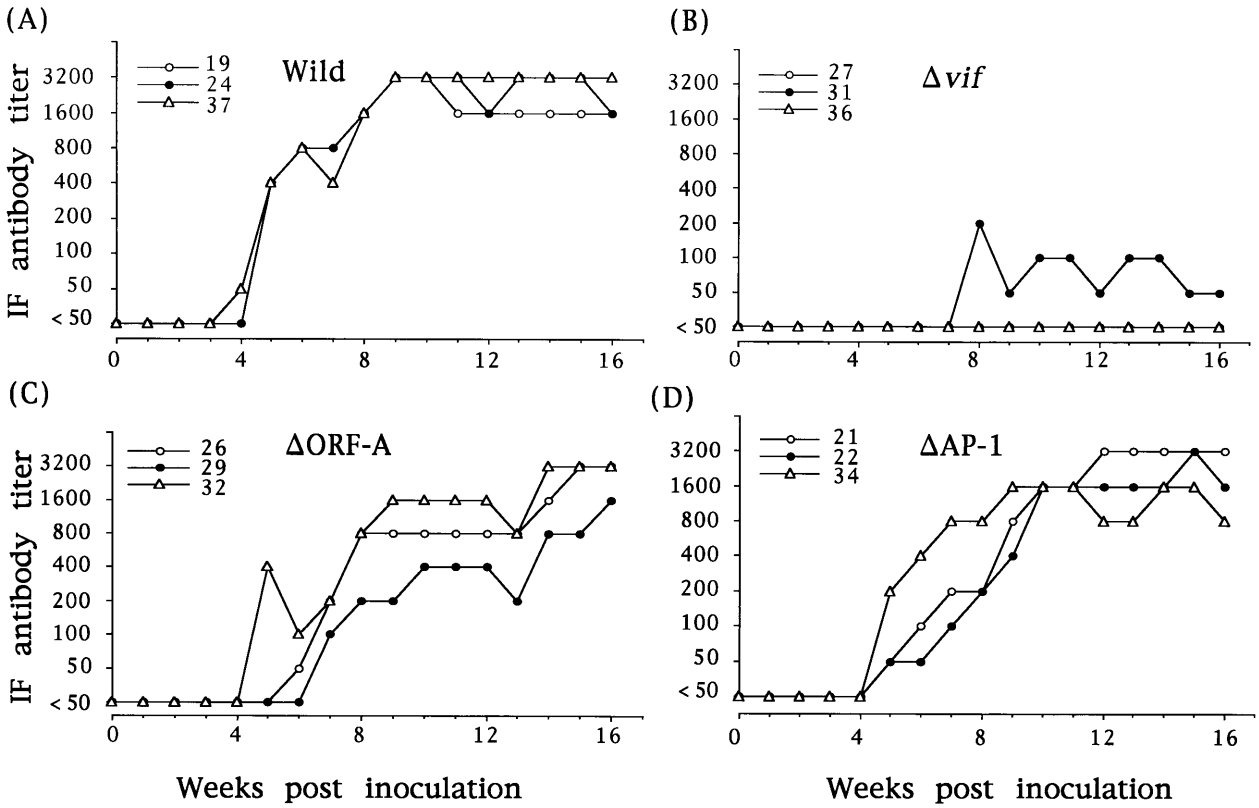

FIG. 1. IF antibody titer. Sera were serially diluted from 1:50 to 1:3,200. Delayed development of antibody compared with the Wild group was observed in ΔORF-A and ΔAP-1 groups. (A) Wild group. (B) Δ*vif* group. The antibody titer rose very weakly in serum from only one of three cats, and no detectable antibody appeared in sera from the other two cats. (C) ΔORF-A group. (D) ΔAP-1 group.

MATERIALS AND METHODS

Construction of mutants. An infectious molecular clone of FIV TM2 strain, termed pTM219 (25, 29) was used as a wild-type virus DNA. All mutants were constructed by recombinant DNA techniques. Constructed DNAs, termed pTM-Ac, pTM-Ac2, and pSTM2D2, are defective in the *vif* and ORF-A genes and the AP-1 binding site, respectively. The pTM-Ac (Δ*vif*) lacks 28 bp between two *Acc*I sites (nucleotides [nt] 5300 and 5328 of TM2 strain). The pTM-Ac2 (ΔORF-A) has a 2-bp insertion at the *Acc*I site (nt 6086). The pSTM2D2 (ΔAP-1) lacks 31 bp between two *Pvu*II sites (nt 85 and 116) which contain the AP-1 binding site together with one of the two AP-4 binding sites. Mutations within DNA constructs were confirmed by sequencing. More detailed descriptions of these mutants were given previously (33, 47, 48).

Cell culture and transfection. CRFK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. MYA-1 cells (31) were maintained in RPMI 1640 growth medium supplemented with 10% fetal calf serum, 100 μg of streptomycin per ml, 100 U of penicillin per ml, 50 μM 2-mercaptoethanol, 2 μg of Polybrene per ml, and 100 U of recombinant human interleukin-2 per ml. The recombinant human interleukin-2 was kindly provided by M. Hattori (Institute for Immunology, Kyoto University, Kyoto, Japan). For transfection, CRFK cells were grown to 90% confluence in a six-well plastic plate and uncleaved plasmid DNA was introduced into the cells by the calcium phosphate coprecipitation method (33, 47, 48).

Animals and inoculations. Fifteen specific-pathogen-free Siamese cats aged from 3.7 to 8.5 weeks were purchased from CSK Research Park Inc. (Tokyo, Japan), and divided into five groups. Before virus inoculation into cats, CRFK cells transfected with plasmid DNAs were monitored for the expression of FIV antigen by an indirect immunofluorescence (IF) assay with a serum sample from a cat infected with FIV. Thereafter, each of the CRFK cells transfected with plasmids (pTM219, pTM-Ac, pTM-Ac2, and pSTM2D2) was adjusted to 3×10^4 FIV antigen-positive cells and then inoculated intraperitoneally into three specific-pathogen-free cats. These groups of cats were designated the Wild (cats 19, 24, and 37), Δ*vif* (cats 27, 31, and 36), ΔORF-A (cats 26, 29, and 32), and ΔAP-1 (cats 21, 22, and 34) groups, respectively. As a negative control, three specific-pathogen-free cats (cats 15, 16, and 18) were inoculated intraperitoneally with uninfected CRFK cells and designated the Control group. All 15 cats were sacrificed at 16 weeks postinoculation (p.i.).

Clinical observations. All cats were observed daily for clinical signs, and body weights and rectal temperatures were measured weekly during the experimental period of 16 weeks. Once a week, blood samples were collected for hematological studies. Leukocytes were counted by the UNOPETTE test 5856 (Becton-Dickinson, Rutherford, N.J.) The total protein was measured with a refractometer (SPR-N; ATAGO, Tokyo, Japan). Levels of gamma globulin, albumin/globulin (A/G) ratios in sera, and CD4 and CD8 subsets of T lymphocytes were measured as described previously (20).

IF antibody titer and virus recovery. Once a week, sera were collected from all 15 cats, and the serum samples were subjected to titer determination for antibody activity by the IF assay with FIV-infected MYA-1 cells. Every 2 weeks, PBMCs from heparinized blood of cats were purified over Ficoll-Paque (Pharmacia, Uppsala, Sweden) by centrifugation. The cells were stimulated with 10 μg of concanavalin A per ml for 3 days and cultured in RPMI 1640 growth medium. One week after cultivation, PBMCs were cocultured with MYA-1 cells and monitored for virus recovery for 7 weeks by the IF assay with a serum sample from a cat infected with FIV.

VN assay. To determine the titer of the virus-neutralizing (VN) antibody, serum samples were heat inactivated at 56°C for 30 min and serially diluted (1:8 to 1:4,096) with RPMI 1640 growth medium prior to the VN assay. Samples (50 μl) of the diluted sera were incubated with an equal volume of 100 50% tissue culture infective doses per 50 μl of FIV for 2 h, and then 100 μl of MYA-1 cells (5×10^5 cells per ml) was added to the mixture. One day after infection, 100 μl of supernatants was removed and 110 μl of fresh medium was added. Culture supernatants were harvested on day 12, and the RT assay was carried out. VN antibody titer was defined as the reciprocal of the highest serum dilution that gave a 50% or greater reduction of viral RT activity when compared with a virus-positive control without serum samples. Stock virus used in the VN assay was obtained from MYA-1 cells infected with supernatant of pTM219-transfected CRFK cells, and its titer was determined by a method described previously (22).

RT assay. Virion-associated RT activities in culture supernatant were measured as follows. A 10-μl volume of culture supernatant was mixed with 50 μl of reaction buffer containing 50 mM Tris-HCl (pH 8.0), 75 mM KCl, 10 mM dithiothreitol, 4.95 mM $MgCl_2$, 0.05 % Nonidet P-40, 10 μg of poly(A) (Pharmacia Biotech, Uppsala, Sweden) per ml as a template, 5 μg of oligo$(dT)_{12-18}$ (Pharmacia Biotech, Uppsala, Sweden) per ml as a primer, and 10 μM [α-^{32}P] dTTP (Du Pont/NEN Research Products). Following a 3-h incubation at 37°C,

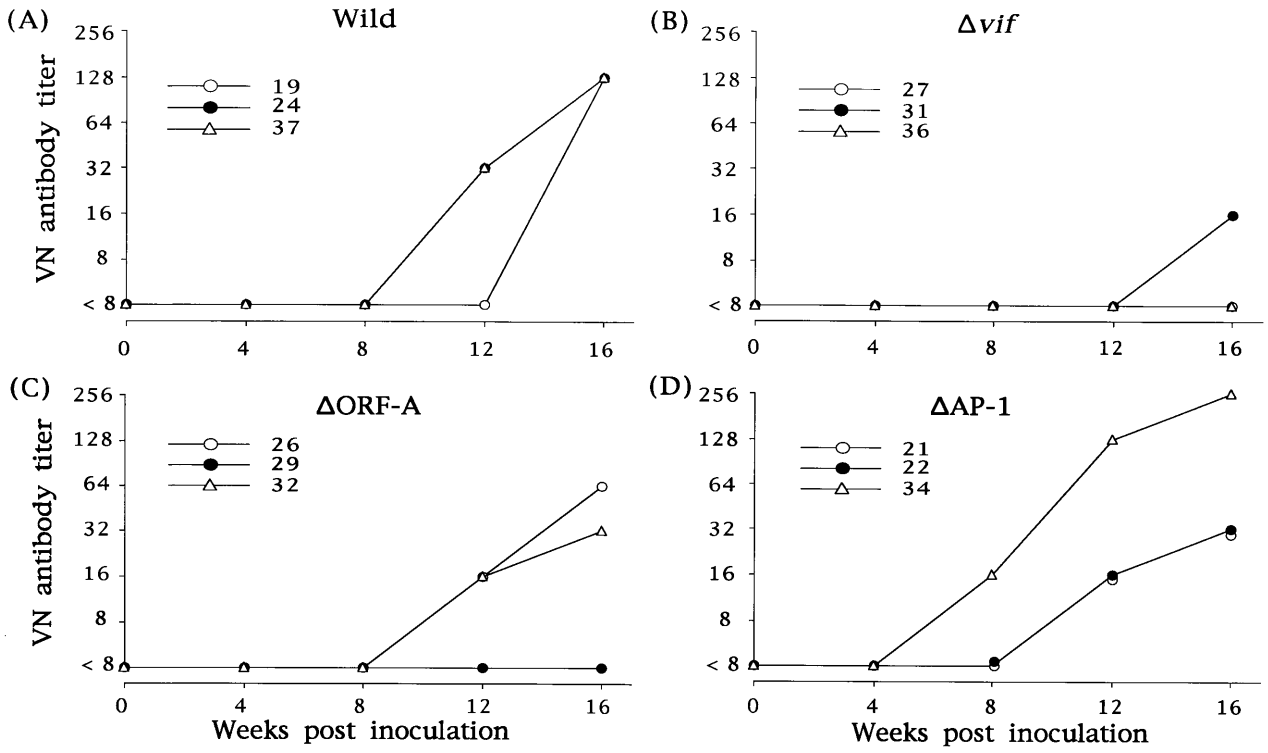

FIG. 2. VN antibody activities. (A) Wild group. (B) Δ*vif* group. (C) ΔORF-A group. (D) ΔAP-1 group.

10 μl of the reaction mixture was spotted directly onto DEAE ion-exchange paper (DE81; Whatman, Maidstone, United Kingdom) and washed three times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to remove unincorporated [α-^{32}P]dTTP. For quantitation of RT activity, spots on DE81 paper were counted with a Bio Imaging Analyzer, BAS 2000 (Fuji Photo Film, Tokyo, Japan).

Quantification of proviral DNA. The viral load of proviral DNA in PBMCs was analyzed by a quantitative PCR method developed previously (20).

Histopathology and immunohistochemistry. Tissue samples were collected at necropsy. Samples of spleen, thymus, and lymph nodes were subjected to histological and immunohistochemical examinations. The lymph nodes sampled included submandibular, retropharyngeal, mesenteric, axillary, and popliteal lymph nodes. Tissues were fixed in 10% neutral-buffered formalin and processed routinely for hematoxylin-eosin (HE) staining. Fixed tissues were sectioned into 30- to 35-μm sections with a microslicer (Douhan EM Co., Osaka, Japan), and the sections were subjected to the labeled streptavidin-biotin method for demonstration of FIV Gag antigen. The sections were preincubated for 1 h in phosphate-buffered saline with 0.1% Triton-X (PBS-T) containing 1% normal goat serum to prevent background staining, washed in PBS-T, and incubated in a freshly prepared solution of 0.5% hydrogen peroxide in absolute methanol for 30 min for inhibition of endogenous peroxidase. The sections were then reacted in a humidified chamber for 24 h with a rabbit anti-FIV Gag serum diluted at 1:3,200 (12, 49). After the mixture was washed in PBS-T, biotinylated goat anti-rabbit immunoglobulin G serum (diluted at 1:800 in PBS-T [Dako A/S, Glostrup, Denmark]) was added, and the mixture was incubated for a further 30 min. Sections were washed in PBS-T, incubated with avidin-biotin peroxidase complex reagent (Dako A/S) for 30 min, washed again in PBS-T, and then incubated in a stirred solution of 3′,3-diaminobenzidine tetrahydrochloride–hydrogen peroxide for 4 min. After further washing in PBS-T, sections were mounted on gelatin-coated slides and counterstained with light green.

Detection of FIV DNA in tissues. The genomic DNA samples from thymus and lymph nodes were extracted with a QIAamp tissue kit (QIAGEN, Chatsworth, Calif.). FIV proviral DNA was detected in these tissue samples by PCR with the primer pair Pr-1 (nt 436 to 455 of the TM2 strain, downstream of LTR) and KA-18 (nt 1066 to 1085, *gag* region). Then, Southern hybridization was carried out as described previously (20).

RESULTS

Clinical observations. During the observation period, all 12 cats infected with various viruses remained asymptomatic. No significant differences in clinical signs, body weights, rectal temperatures, leukocyte counts, levels of total protein and gamma globulin, and A/G ratios were observed among cats in each group (data not shown).

IF and VN antibody titers. IF antibodies against FIV were sequentially measured by the IF assay. The kinetics of antibody titers in each cat in four groups is shown in Fig. 1. IF antibodies were detected first in serum obtained from a cat (cat 37) in the Wild group as early as 4 weeks p.i. All the cats infected with various viruses developed IF antibodies in sera except two cats in the Δ*vif* group (cats 27 and 36), which showed no antibody response throughout the observation period. IF antibody titers in cats in the Wild, ΔORF-A, and ΔAP-1 groups increased progressively from 4 or 5 weeks p.i. The titers in the three cats in the Wild group reached 1:3,200 at 9 weeks p.i., whereas those in the cats in the ΔORF-A and ΔAP-1 groups increased more slowly than did those in the Wild group. A titer of only 1:200 developed in cat 31 in the Δ*vif* group at 8 weeks p.i. No antibody response was observed in cats in the Control group.

VN antibody responses in serum samples were also examined (Fig. 2). VN antibodies were detected from all but three cats infected with the viruses (cat 27 and 36 in the Δ*vif* group and cat 29 in the ΔORF-A group). Of these three cats, the two (cats 27 and 36) in the Δ*vif* group were also negative for IF antibodies during the observation period; however, cat 29 in the ΔORF-A group had an IF antibody titer as high as 1:1,600 at 16 weeks p.i.

Viral load in PBMCs. Quantitative PCR was performed to quantitate proviral DNA copies in 10^5 PBMCs from cats infected with various viruses. FIV proviral DNAs were detected from 1 week p.i. in all of the FIV-infected groups. The numbers of proviral DNA copies in cats in the Wild group increased progressively during the observation period and were larger than those in cats in the other groups. The copy numbers

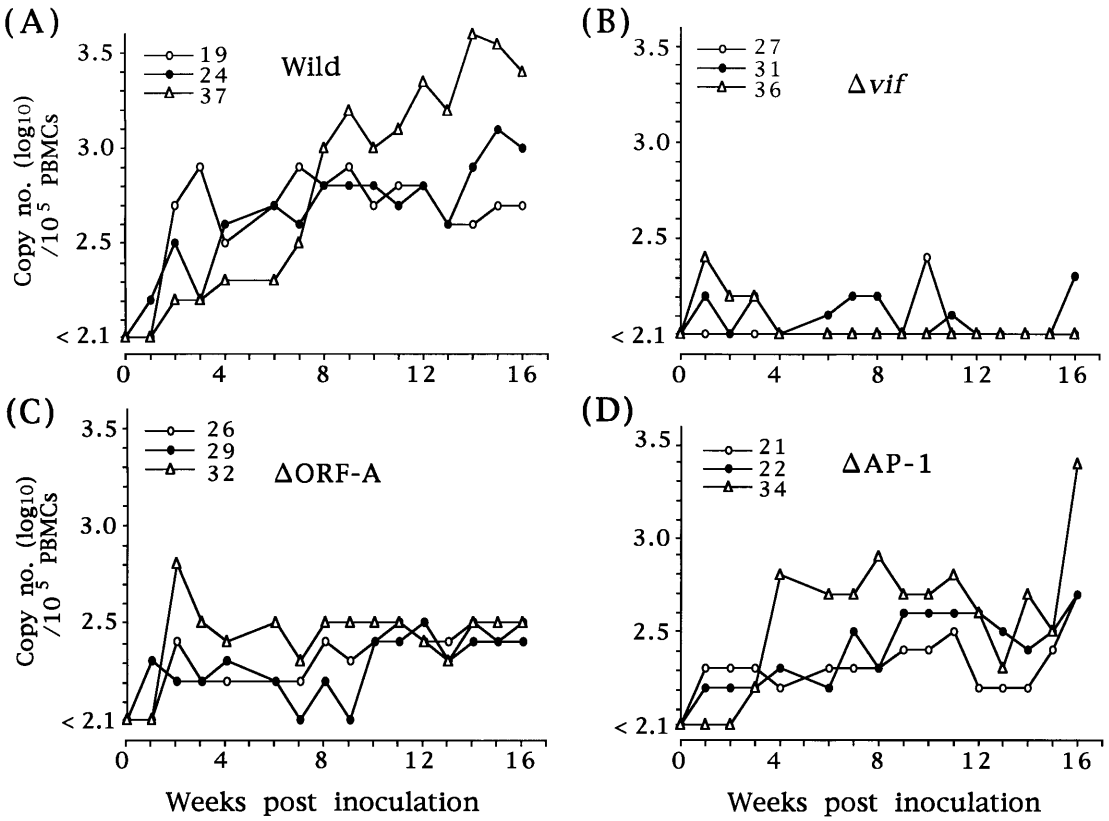

FIG. 3. Viral load of FIV proviral DNA in 10^5 PBMCs. Proviral copy number was quantified as described previously (20). (A) Wild group. (B) Δ*vif* group. (C) ΔORF-A group. (D) ΔAP-1 group.

for the ΔORF-A and ΔAP-1 groups seemed to be kept at relatively low levels (Fig. 3), and those for the Δ*vif* group were very low. Although the copy numbers in cat 31 in the Δ*vif* group, from which IF and VN antibodies were detected, reached $10^{2.3}$ copies in 10^5 PBMCs at 16 weeks p.i., those in the other two cats (cats 27 and 36) in the Δ*vif* group were less than the level of detection of $10^{2.1}$ copies in 10^5 PBMCs at the week.

Virus recovery. Viruses were recovered from PBMCs of all cats in the Wild, ΔORF-A, and ΔAP-1 groups but not from those in the Control and Δ*vif* groups during the observation period (Table 1). However, the frequencies of virus recovery from cats in the ΔAP-1 and ΔORF-A groups were not as high as those in the Wild group. In the Δ*vif* group, although both IF and VN antibodies were detected from one cat (cat 31) and proviral DNAs were detected from all cats, no virus was recovered.

Lymphocyte subsets. CD4 and CD8 subsets in all 15 cats were measured every week until 15 weeks p.i. by flow cytometric analysis. The CD4/CD8 ratios in cats in the Wild and ΔORF-A groups decreased in comparison with those measured preinoculation, and those in two cats in the ΔAP-1 group decreased slightly, but no significant reduction of the ratio was observed in cats in the Control and Δ*vif* groups (Fig. 4A to E). The preinoculation mean CD4/CD8 ratios in the three cats in the Wild, Δ*vif*, ΔORF-A, ΔAP-1, and Control groups were 1.65, 1.66, 1.64, 1.55, and 1.65, respectively. At 15 weeks p.i., the ratios in the Wild, Δ*vif*, ΔORF-A, ΔAP-1, and Control groups were 1.07, 1.52, 1.18, 1.30, and 1.52, respectively (Fig. 4F).

Histopathology and immunohistochemistry. The results of histopathological and immunohistochemical examinations with anti-FIV Gag antibody are summarized in Table 2. Histological changes associated with virus inoculation were found in the

TABLE 1. Virus recovery

Group	Cat no.	Age (mo)	Sex[a]	Virus	Virus recovery at wk p.i.: 1	3	5	7	9	11	13	15
Control	15	8.5	M	None	−	−	−	−	−	−	−	−
	16	8.5	M	None	−	−	−	−	−	−	−	−
	18	7.3	M	None	−	−	−	−	−	−	−	−
Wild	19	7.5	F	pTM219	+	+	+	+	+	−	−	+
	24	6.0	F	pTM219	+	+	+	+	+	+	−	−
	37	3.7	F	pTM219	+	+	−	−	−	+	+	−
Δ*vif*	27	6.0	M	pTM-Ac	−	−	−	−	−	−	−	−
	31	5.7	M	pTM-Ac	−	−	−	−	−	−	−	−
	36	3.7	F	pTM-Ac	−	−	−	−	−	−	−	−
ΔORF-A	26	6.0	M	pTM-Ac2	−	+	−	+	−	−	−	−
	29	5.7	M	pTM-Ac2	−	−	−	+	−	−	−	+
	32	5.5	F	pTM-Ac2	+	+	−	−	−	−	−	−
ΔAP-1	21	7.2	M	pSTM2D2	−	−	+	+	−	+	−	−
	22	7.2	M	pSTM2D2	−	+	−	+	+	−	−	+
	34	5.5	M	pSTM2D2	−	+	−	−	+	−	−	−

[a] M, male; F, female.

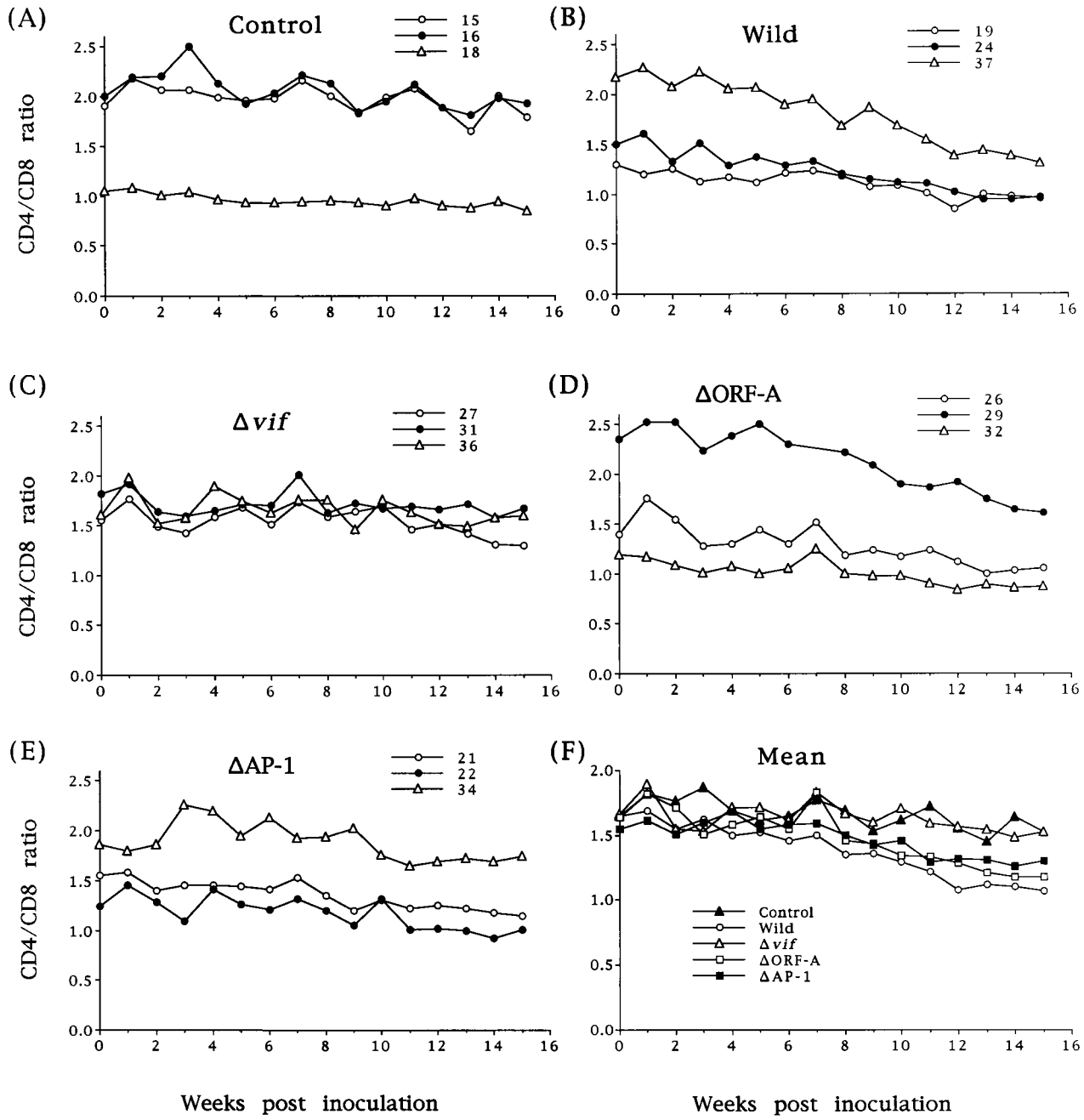

FIG. 4. Changes in the CD4/CD8 ratio. (A) Control group. (B) Wild group. (C) Δ*vif* group. (D) ΔORF-A group. (E) ΔAP-1 group. (F) Mean ratios in each group. At 15 weeks p.i., mean ratios had decreased by 0.58 (Wild group), 0.46 (ΔORF-A group), and slightly decreased 0.25 (ΔAP-1 group) compared with the preinoculation ratios. However, significant changes were not observed in the Control (0.13 decrease) and Δ*vif* (0.14 decrease) groups.

lymph nodes, spleen, and thymus. The lymph nodes of nine cats (one in Δ*vif*, two in ΔORF-A, and three each in the Wild and ΔAP-1 groups) showed distinctive enlargement of the germinal centers and follicular fragmentation (Fig. 5B). The lymph follicles and germinal centers of the spleen were enlarged in five cats (three in the Wild group and two in ΔAP-1 group) (Fig. 5D). The germinal centers were composed mainly of large lymphocytes and reticular cells and were surrounded by a thin, dark mantle zone of small lymphocytes. In two cats (cats 37 and 34 in the Wild and ΔAP-1 groups, respectively), the cortex of the thymus appeared lighter than that of others, probably because of an increased population of lymphoblasts. Interestingly, in the Δ*vif* group, histological changes in lymph nodes were found only in cat 31, which had both IF and VN antibody responses. On the other hand, no histological changes were found in cats 27 and 36 in the Δ*vif* group, which had neither IF nor VN antibody responses.

FIV Gag antigen was found in several cells of the lymph nodes of all cats infected with viruses except for one (cat 36) in the Δ*vif* group (Fig. 5E; Table 2). Most of the antigen-positive cells were considered to be follicular dendritic cells (FDCs) characterized by dendritic cytoplasmic projections. No immu-

TABLE 2. Histopathological, PCR and immunohistochemical observations in tissues

Group	Cat no.	Histological change[a]			PCR		FIV Gag antigen[b]	
		Lymph nodes (EGC)[c]	Spleen (EGC)	Thymus (lymphoblasts)	Lymph nodes	Thymus	Lymph nodes	Thymus
Control	15	−	−	−	−	−	−	NE
	16	−	−	−	−	−	−	−
	18	−	−	−	−	−	−	NE
Wild	19	++	+	−	+	+	+	NE
	24	+	+	−	+	+	+	+
	37	+	+	+	+	+	+	−
Δ*vif*	27	−	−	−	−	−	+	−
	31	+	−	−	+	−	+	+
	36	−	−	−	−	−	−	+
ΔORF-A	26	−	−	−	+	+	+	NE
	29	+	−	−	+	+	+	−
	32	+	−	−	+	+	+	NE
ΔAP-1	21	+	−	−	+	+	+	NE
	22	+	+	−	+	+	+	NE
	34	+	++	+	+	+	+	NE

[a] Histological changes were scored as negative (−), positive (+), or severe (++).
[b] FIV Gag antigen-positive cells were scored as negative (−), positive (+), or not examined (NE).
[c] EGC, enlargement of the germinal centers.

noreactivity was found in the spleen in any of the cats. When the thymus was immunostained in seven cats, three (cat 24 of the Wild group and cats 31 and 36 of the Δ*vif* group) showed positive reactions in several epithelial reticular cells in the medulla of the thymus.

Detection of FIV DNA in tissues. FIV proviral DNA was detected from the lymph nodes and thymus of all cats infected with viruses except those in the Δ*vif* group (Table 2). In cats in the Δ*vif* group, proviral DNA was detected only from lymph nodes of cat 31, in which IF and VN antibody activities were developed and histopathological changes in lymph nodes were observed. The results of PCR analysis were generally correlated with the results of immunological and histopathological observations and viral loads in PBMCs.

DISCUSSION

The auxiliary genes of lentiviruses are supposed to be one of the determinants of disease progression in vivo. The genes of SIV have been studied extensively, and the *nef* gene but not the *vpr* gene of SIV was shown to be required for efficient virus replication and disease progression (14, 18, 24, 26). Enhancer elements in the LTR are also considered to play a role as determinants of pathogenesis in vivo. Experiments with mutants of murine leukemia virus revealed that the disease specificity of various murine leukemia viruses is controlled by the nature of the enhancer elements and is associated with high transcriptional activity of the LTR in cells (3, 7, 27). The pathogenesis of FIV infection in cats might be also attributable to both its auxiliary genes and its enhancer elements. However, it is still unknown whether they are indeed involved in viral replication and pathogenicity in vivo. In the present study, we demonstrated that the *vif* and ORF-A genes and the AP-1 binding site of FIV are required to allow efficient viral replication in the early stage of infection in cats.

All known lentiviruses except equine infectious anemia virus encode *vif* gene (36). Moreover, *vif* genes from HIV-1, HIV-2, and SIV have cross-complementation ability (39, 42). These observations might imply an important role of the *vif* gene in the natural host. In the present study, we showed that Δ*vif* virus could replicate in lymph nodes, where the virus can be transmitted in a cell-to-cell manner, but it could neither replicate well in circulating PBMCs nor affect immunological responses and histological changes in cats. The loss of infectivity and cell-type-specific phenotype of Δ*vif* virus (40, 48) might result in the severely reduced viral replication in cats. In the study of HIV-1, sequential analysis of quasispecies from a long-term survivor infected with HIV-1 revealed the presence of inactivating mutations in the auxiliary genes including the *vif* gene (30). Similar to the cats in the Δ*vif* group, the patient had been negative for HIV isolation and kept a very low viral burden for 13 years since infection. Our findings, together with this report, suggest that the *vif* gene of lentivirus plays a crucial role in viral replication and probably pathogenesis in vivo. Additionally, it is of interest that although cats in the Δ*vif* group had a very low viral load, FIV Gag antigen was observed in FDCs in lymph nodes. It was reported that in HIV infection, FDCs in lymphoid tissue contain highly infectious HIV and can convert neutralized HIV into an infectious form even in the presence of an excess amount of VN antibody (16). Our results might also suggest that FIV can replicate well in FDCs and that FDCs in lymph nodes act as a major reservoir and/or a primary target of FIV in the early stage of infection in cats. Recently, it was demonstrated that the Vif protein of HIV is incorporated into virus particles and the intracellular localization of Vif protein is dependent on the presence of the intermediate filament vimentin (21). Presumably, Vif protein has a function as a virion component either by regulating virus maturation or following virus entry into host cells, possibly involving an interaction with the cell-type-specific cellular cytoskeletal network.

In primate lentiviruses, a *trans*-activator gene called *tat* (related to the ORF-A gene of FIV in its location on the viral genome) is important for transcriptional transactivation of their LTRs and is basically indispensable for viral replication. In the present study, we demonstrated that cats infected with

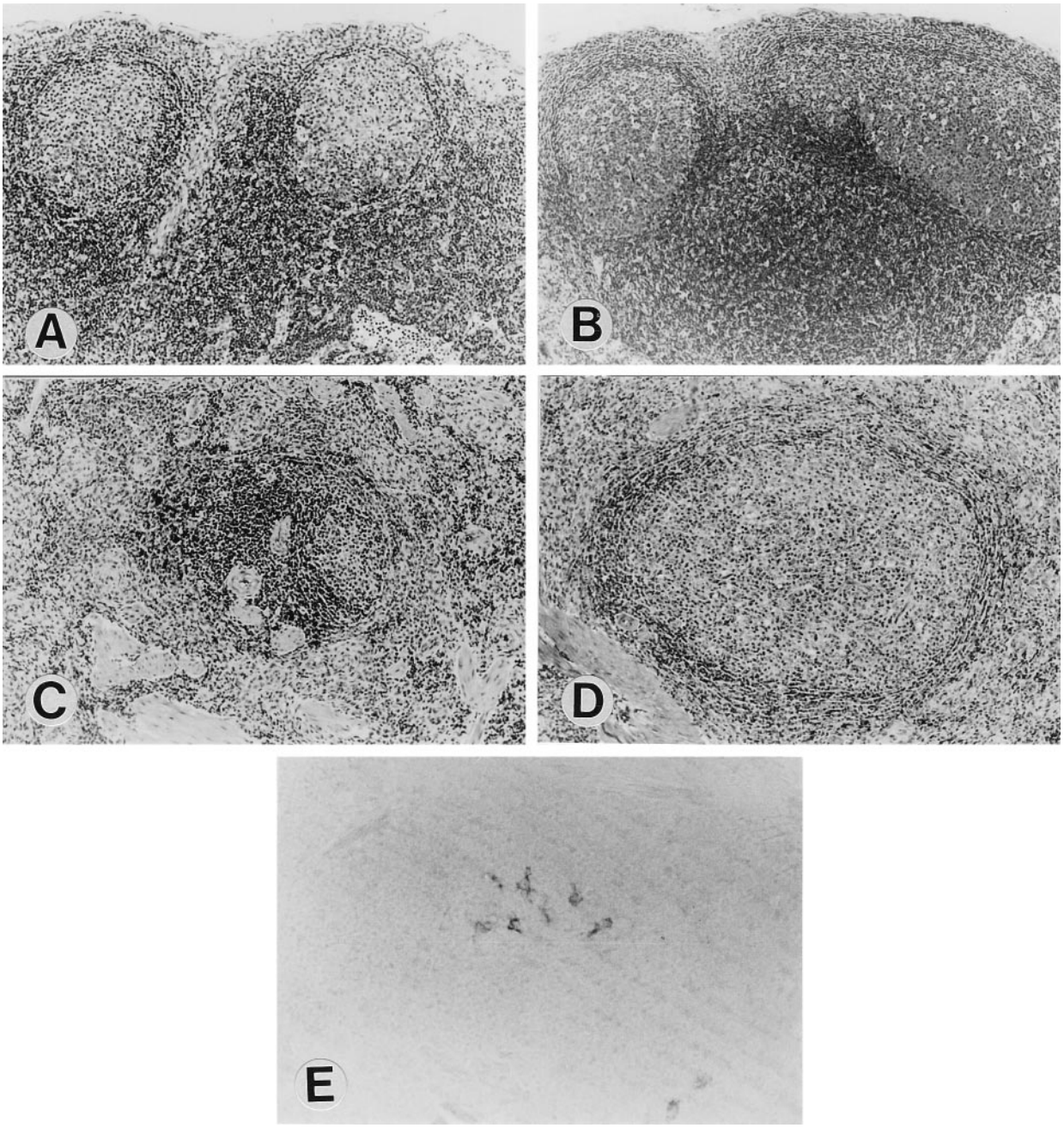

FIG. 5. Histopathological and immunohistochemical findings. (A) Normal appearance of germinal centers of the lymph node in the Control group. HE stain. Magnification, ×95. (B) Enlarged germinal centers and follicular fragmentation of the lymph node. Wild group. HE stain. Magnification, ×95. (C) Normal appearance of lymph follicle with eccentric, small germinal centers of the spleen. Control group. HE stain. Magnification, ×125. (D) Enlarged germinal center with thin mantle of small lymphocytes of the spleen. ΔAP-1 group. HE stain. Magnification, ×125. (E) Immunopositive dendritic cells in the lymph node. ΔORF-A group. Labelled streptavidin-biotin stain. Magnification, ×190.

ΔORF-A virus (ΔORF-A group) showed slower development of antibodies, lower viral loads, milder reduction of CD4/CD8 ratios, and milder histological changes than did cats in the Wild group. These changes were smaller than those in cats in the Δ*vif* group but more severe than those of cats in ΔAP-1 group. Similar to our observations, Sparger et al. (43) reported that over 50% of cats inoculated with a cloned FIV 34TF10, which contains a stop codon in the ORF-A gene, failed in seroconversion, virus recovery, and detection of proviral DNA by PCR. Moreover, these cats had a lower viral load and milder reduction of CD4/CD8 ratio than did cats infected with FIV which contains an intact ORF-A gene. There seems to be a good correlation between the reduced efficiency of viral replication in vitro and lower viral loads with milder immunological and histological effects in vivo (38, 47). Our results suggest that the ORF-A gene of FIV is dispensable for the viral life cycle but plays an important role in efficient viral replication in vivo. Similar to FIV, the caprine arthritis encephalitis virus *tat* gene is also dispensable for viral replication both in vitro and in vivo, and the Tat protein of caprine arthritis encephalitis virus also

weakly transactivates its LTR (15, 17). Recently, another role was reported for Tat protein in HIV-1 and HIV-2, where the protein is involved in virion infectivity as well as transcriptional activation (19). Indeed, Luznik et al. demonstrated that *tat*-defective HIV-1 can replicate in certain monocytic and T-cell lines (U937 and MT-4 cells) in vitro (28). These results suggest that the FIV ORF-A gene might act on virion infectivity but not transcriptional transactivation activity in vivo.

We initially expected that viral loads of ΔAP-1 virus and immunological and histological changes in cats infected with ΔAP-1 virus would be almost the same as those in cats infected with wild-type virus, based on the results of in vitro studies with T-cell lines and PBMCs (32, 33). Contrary to our expectations, ΔAP-1 virus did not grow as efficiently as wild-type virus. The different results of in vivo and in vitro studies might be due to the following possibilities. The efficiency of the replication of ΔAP-1 virus might differ both in cell types and at activation stages of the cells infected with the virus. FIV can infect various cell types in vivo, such as monocytes/macrophages, immunoglobulin-positive cells, brain cells, and other cells, as well as T lymphocytes (1, 2, 8, 11, 49). Basal promoter activity of the FIV LTR is also dependent on cell types (33, 44, 45). Furthermore, cellular factors such as Jun and Fos, which bind to the AP-1 binding site to activate LTRs, are induced in accordance with differentiation and activation of the target cells by immune responses in individuals (13, 41). However, ΔAP-1 virus cannot be affected by the factors. Our observations suggest that the replication of ΔAP-1 virus in cats greatly differed both in cell types and at activation stages of infected cells, and the AP-1 binding site is responsible for full-blown viral replication in vivo.

In this study, we showed that all three mutants still retain infectivities in cats with different growth kinetics. If a mutant virus could induce high levels of VN antibody and cytotoxic T-lymphocyte activities without showing any diseases in cats, the virus might be a candidate for a live attenuated vaccine to protect cats from FIV infection. Further studies are required to investigate the roles of the genes and enhancer elements of FIV in vivo for understanding the pathogenesis of FIV and vaccine development.

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