

## Effects of insertion of multiple AP-1 binding sites into the U3 region of the long terminal repeat of feline immunodeficiency virus

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**Summary.** An oligonucleotide containing multiple AP-1 binding sites was introduced into the regulatory sequence in the long terminal repeat (LTR) of feline immunodeficiency virus (FIV). Chloramphenicol acetyltransferase assay revealed that basal promoter activity of the mutated LTR was higher than that of the wild-type LTR in Crandell feline kidney (CRFK) cells. The mutated LTR was introduced into an infectious molecular clone of FIV and the clone was transfected into CRFK cells. The virus production of the mutant in the cells was as high as that of the wild-type when determined by the reverse transcriptase activity assay. The growth of the mutant virus obtained from the transfected CRFK cells was examined in feline T lymphoblastoid cell lines (MYA-1 and FeL-039 cells) and primary feline peripheral blood mononuclear cells (fPBMCs). The growth was delayed when compared with that of the wild-type virus in all the cells used. Upon examination by polymerase chain reaction, the length of the LTR of the mutant virus was shortened in both MYA-1 cells and fPBMCs. Sequence analysis revealed that the insertion was completely deleted 39 days after infection in the MYA-1 cells.

### Introduction

Feline immunodeficiency virus (FIV) is the etiological agent of an acquired immunodeficiency syndrome-like disease in cats [24, 32]. FIV is a member of the genus *Lentivirus* in the family *Retroviridae* [1]. Lentivirus expression is regulated by virally encoded proteins and by cellular transcriptional factors [2, 20]. Like other lentiviruses, FIV contains the *rev* gene [12, 25], however it is not clear whether the virus contains a *trans*-activator gene [19, 28]. Also in the U3 region of FIV LTR,

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several putative binding sites for enhancer proteins such as AP-1, AP-4, C/EBP are present [13, 14, 26].

Previously we reported that by deletion of AP-1 and AP-4 related sequences in the LTR, promoter activity was reduced in Crandell feline kidney (CRFK) and *felis catus* whole foetus-4 (fcwf-4) cells using the chloramphenicol acetyltransferase (CAT) assay [11, 19]. Recently, Thompson et al. [29] reported that protein(s) present in the nuclear extract from feline T cells (F422 cells) which are permissive for FIV replication bind to the putative AP-1 and AP-4 binding sites. These findings suggest that the AP-1 in feline cells can bind to the AP-1 binding site in the LTR and is critical for the basal promoter activity of the LTR. Conversely, a mutant virus which lacks the AP-1 and AP-4 binding sites replicated in feline T lymphoblastoid cell lines (MYA-1 and FeL-039 cells) [19] and primary feline peripheral blood mononuclear cells (fPBMCs) [17]. These data indicate that the AP-1 and AP-4 binding sites are not necessary for the efficient replication of FIV in feline T lymphocytes.

In this study, we introduced an oligonucleotide containing multiple AP-1 binding sites adjacent to the original AP-1 binding site in the U3 region of the LTR and examined the effects of the insertion on the promoter and growth activities of the mutant.

## Materials and methods

### *Cell culture*

CRFK cells [3] and fcwf-4 [9] were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum (FCS). fPBMCs obtained from a specific pathogen-free cat were isolated as described previously [15, 16]. MYA-1 [16, 21] and FeL-039 [30] cells, and fPBMCs were maintained in the RPMI 1640 growth medium supplemented with 10% FCS, antibiotics, 50  $\mu$ M 2-mercaptoethanol, 2  $\mu$ g/ml polybrene and 100 units/ml of recombinant human interleukin-2 at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

### *DNA constructs*

The CAT constructs under the control of the LTRs of FIV, termed pSPTM1CAT and pSPTM1A PvCAT, were described previously [10, 19]. They were made by placing FIV LTR from an infectious clone of FIV TM1 strain (pFTM191CG) [14] and its deleted mutant in front of the CAT gene of pSPCAT which has both CAT gene and poly A signal [19]. An infectious clone of FIV, termed pSTM2, and a mutated infectious clone which lacks a 31 base pair fragment containing one AP-1 and one AP-4 binding sites, termed pSTM2D2, were described previously [19]. The pSTM2 5' and 3' LTRs were derived from TM2 and TM1 strains of FIV, respectively [19]. After digestion of pSTM2 with *SacI*, a small fragment which contained the 5' LTR of pSTM2 was subcloned into the *SacI* site of pSP72 vector (Promega, Madison, WI, U.S.A.) and designated as pSTM2LTR. To introduce an oligonucleotide containing multiple AP-1 binding sites into pSTM2LTR, oligonucleotides (5'-CTAGCATGAGTCAGAGTTATGAGTCAGAGT-3' and 5'-CTAGACTCTGACTCATAACTCTGACTCATG-3') were synthesized. The oligonucleotides were annealed, phosphorylated by T4 DNA kinase and then ligated into *NheI* site of pSTM2LTR, and designated as pSTM2-5APLTR. The sequence of the pSTM2-5APLTR was determined by the cycle sequencing method using dye-terminator supplied by Applied Biosystems (ABI) (Foster City, CA, U.S.A.). To generate pSTM2 (5AP, 5') which has the mutated LTR at the 5' end of the FIV genome of pSTM2, the *SacI* fragment of pSTM2 which contains the 5' LTR was substituted with the *SacI* fragment of pSTM2LTR-5AP. To generate pSTM2-5AP which has the mutated LTR on both

sides of the genome, a *Bam*HI fragment of the pSTM2 (5AP, 5') was introduced into the *Bam*HI site of pSTM2-5APLTR. To construct pSTM2-5APCAT, the *Sac*I-*Nar*I fragment of pSTM2-5APLTR was cloned into the pSPCAT. pVisLTRCAT [7] which is a visna virus LTR CAT reporter plasmid was kindly provided by Dr. J. E. Clements (The Johns Hopkins University, MD, U.S.A.).

#### *Transfection of plasmid DNA*

For transfection of plasmid DNA into CRFK and fcwf-4 cells, cells were plated in six-well dishes one day before transfection. Plasmid DNAs were transfected by the calcium phosphate coprecipitation method [6, 31]. Four hours after transfection, the cells were washed with phosphate-buffered saline (PBS), shocked with glycerol and then placed in fresh medium.

#### *CAT assay*

For the CAT assay, cell monolayers in each well of six-well dishes were harvested by scraping 48 h after transfection. After being washed once with PBS, the cells were lysed by freezing and thawing four times in 250 mM-Tris-HCl pH 7.8. Cell debris was pelleted by centrifugation for 5 min at 4 °C and various amounts of each extract were assayed for CAT activity [5] by the solvent partition method [22]. In brief, a 240 µl reaction mixture containing 100 mM-Tris-HCl pH 7.8, 1.0 mM-chloramphenicol, 3.7 kBq of [<sup>14</sup>C] acetylcoenzyme A (Du Pont, NEN), and cell extract was overlaid with 5 ml of scintillation fluid (EconofluorII; Du Point, NEN). The reaction was carried out at 37 °C and production of radioactively labeled acetylchloramphenicol was monitored by counting in a liquid scintillation counter. The CAT activity of each promoter plasmid was presented as the net d.p.m. of product formed/h. All the CAT assay data reported in this paper are from the points in the linear range of the assay.

#### *Virus infection*

MYA-1 and FeL-039 cells, and fPBMCs ( $1.5 \times 10^6$  cells) were infected with the FIVs derived from the infectious molecular clones. The cells were seeded at  $3 \times 10^5$  cells/ml in growth medium. The cell numbers were adjusted to  $3 \times 10^5$ /ml in fresh growth medium at the indicated time.

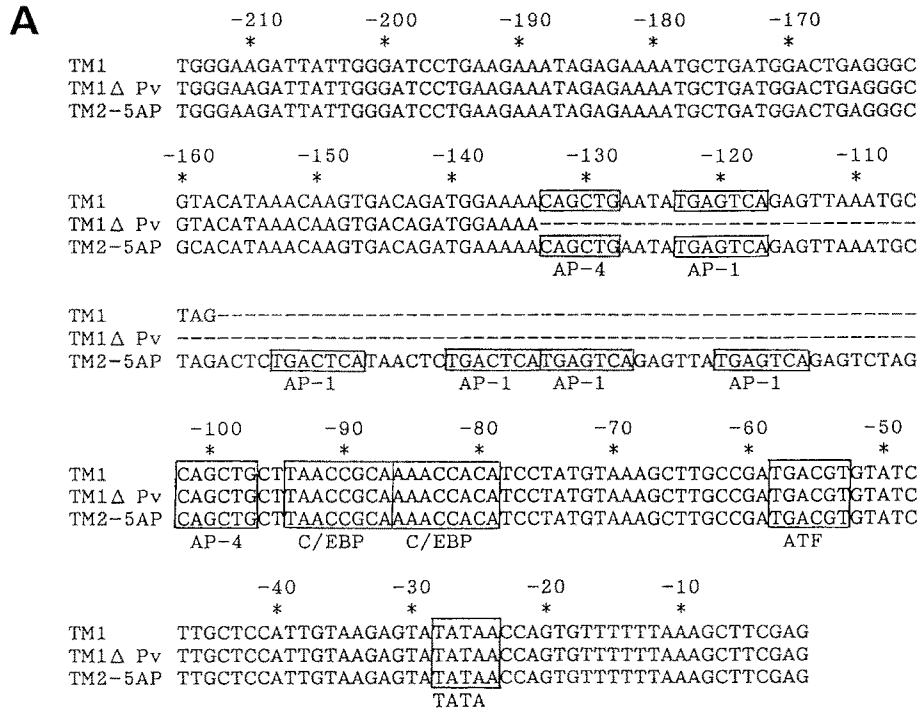
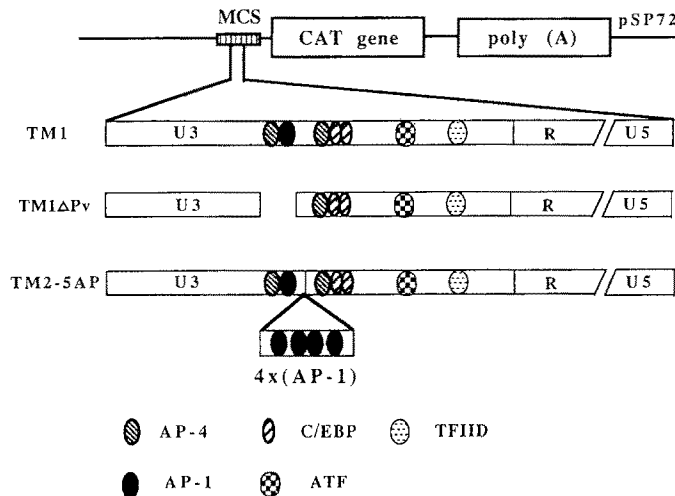
#### *Reverse transcriptase (RT) activity assay*

The Mg<sup>2+</sup>-dependent RT activity in cell culture supernatants was assayed as described previously [23].

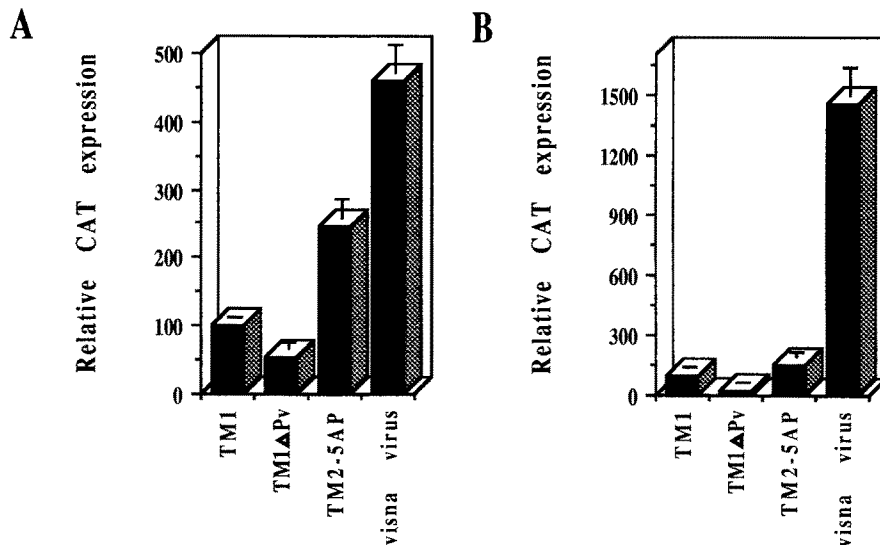
#### *Polymerase chain reaction (PCR) and sequence analyses*

For amplification of FIV LTRs, an antisense primer (N1) (5'-GTCCCTGTTTCGGGCGCCAACT-3', nucleotide 381–361) and a sense primer (N2) (5'-GATGGCAAATCTAGAGAACCGC-3', nucleotide 9011–9032) were synthesized corresponding to the primer binding site and an upstream sequence from the polypurine tract of FIV, respectively. The sequences of primers originated from the sequence of FIV TM2 strain [13]. PCR was carried out by the method of Saiki et al. [27] in a 50 µl volume overlaid with an equal volume of mineral oil. A GeneAmp PCR Reagent kit (Perkin Elmer Cetus, Norwalk, CT U.S.A.) was used for the reactions. Amplification proceeded for 30 cycles in DNA thermal cycler model PJ2000 (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). One cycle consisted of incubations at 94, 64, and 72 °C for 1, 1, and 2 min, respectively. After amplification, a 5 µl sample of the 50 µl-reaction was electrophoresed on a 1.5% agarose gel (in Tris-borate-EDTA buffer).

Amplified DNA digested with *Xba*I and *Nar*I was subcloned into pUC118 and then used for sequence analysis. The double stranded DNA was annealed with N1 or N2 primer, extended by the cycle sequencing method using dye-terminator supplied by ABI, and then analyzed by a model 370A ABI autosequencer.

**B**

**Fig. 1. A** Nucleotide sequence of the U3 region of the plasmids pSPTM1 (*TM1*), pSPTM1 Δ Pv (*TM1Δ Pv*), and pSTM2-5AP (*TM2-5AP*). The recognition sequences of enhancer and promoter proteins and transcription initiation signal (TATA box) are boxed: *AP-1* (TGA[C/G]TCA), *AP-4* (CAGCTG), *C/EBP* ([T/A]AACC[A/G]CA), *ATF* (TGACGT), and TATA box (TATAA). **B** Schematic view of the reporter plasmids of the FIV LTR and its mutants. Abbreviations: *MCS* multicloning site; *poly (A)* poly (A) signal



**Fig. 2.** Basal promoter activity of FIV LTR, its mutants and visna virus LTR. Each reporter plasmid was transfected into CRFK (A) and fcwf-4 (B) cells. Two days after transfection, the CAT product in the cells was monitored. After subtraction of the background level obtained by pSPCAT (promoter less reporter plasmid), CAT expression relative to the level obtained from pSPTM1 is shown. Three independent experiments were performed, and the average and the standard deviation are presented

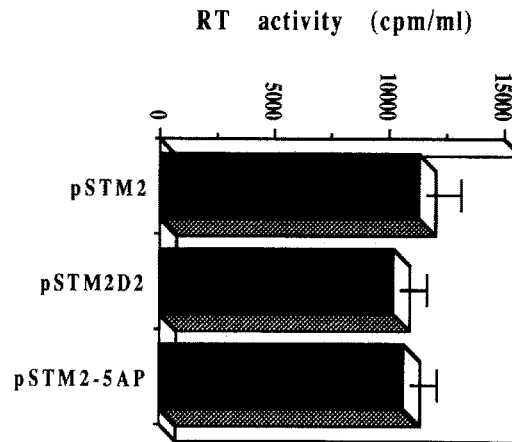
## Results

### *Insertion of multiple AP-1 sites into the FIV LTR*

Oligonucleotides containing two AP-1 sites were synthesized and inserted into the *Nhe*I site of FIV LTR (pSTM2LTR) to generate pSTM2-5APLTR. Sequence analysis revealed that the pSTM2-5APLTR has five AP-1 binding sites (Fig. 1A). The insertion of the plasmid was stable in *E. coli*. The mutated LTR containing five AP-1 sites was placed upstream from the CAT gene and poly A signal of pSPCAT to construct pSTM2-5APCAT. The sequence of the U3 region of pSPTM1CAT, pSPTM1 $\Delta$ PvCAT and pSTM2-5APCAT is shown in Fig. 1A, and the schematic view of the LTR CAT reporter plasmids is shown in Fig. 1B. The LTR of TM2 strain differed from that of TM1 strain only at positions -138 (G to A) and -159 (T to C) [13, 14], and both the LTRs of TM1 and TM2 showed almost the same promoter activity in fcwf-4 and CRFK cells (unpubl. data).

### *Effects of the insertion of AP-1 sites on basal promoter activity and viral replication*

Two  $\mu$ g of pSTM2-5APCAT, pSPTM1CAT, pSPTM1 $\Delta$ PvCAT, pSPCAT or pVisLTRCAT were transfected into CRFK and fcwf-4 cells. Forty-eight hours after transfection, the CAT products of the clones were measured by the solvent partition method. The basal promoter activity of the pSTM2-5APCAT was significantly higher than that of the pSPTM1CAT in CRFK cells (Fig. 2A), but not in fcwf-4 cells



**Fig. 3.** RT productions of the FIV infectious molecular clone (*pSTM2*) and its mutants (*pSTM2D2* and *pSTM2-5AP*) in the transfected CRFK cells. Three independent experiments were performed, and the average and the standard deviation are presented

(Fig. 2B). In addition, the activity of the pSTM2-5APCAT was lower than that of the pVisLTRCAT in both cell types (Fig. 2).

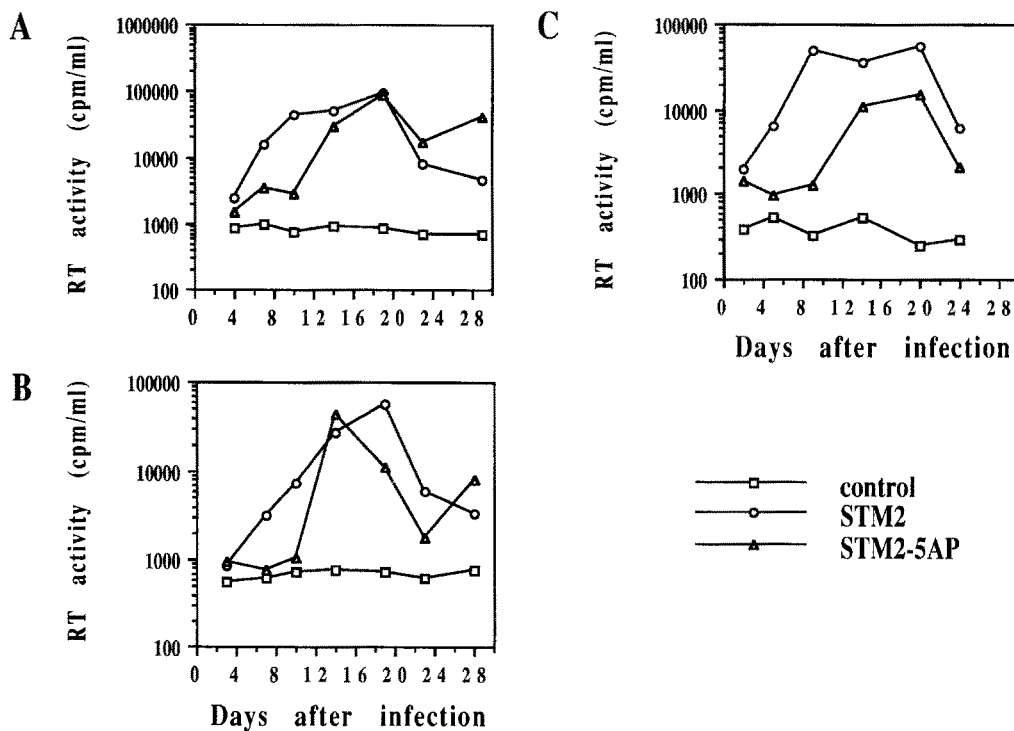
The mutated LTR was introduced into an infectious molecular clone of FIV (termed pSTM2) and designated pSTM2-5AP. Five  $\mu$ g of pSTM2 (wild-type), pSTM2D2 (AP-1-deleted mutant) or pSTM2-5AP were transfected into CRFK cells, and three days after transfection, the virus production in the culture supernatant was measured using the RT activity assay. As shown in Fig. 3, the RT activity of the pSTM2-5AP was similar to those of pSTM2 and pSTM2D2.

Samples of the culture supernatants containing equivalent radioactivity from the CRFK cells transfected with the respective infectious molecular clones were inoculated into MYA-1 cells, FeL-039 cells and fPBMCs, and the virus production was monitored by the RT activity assay. As shown in Fig. 4, the growth of the virus derived from pSTM2-5AP was delayed in all the cells, however, the peaks of the RT activities of both viruses occurred at similar levels in either MYA-1 (Fig. 4A) or FeL-039 (Fig. 4B) cells. On the other hand, the peak of RT activity of the virus from pSTM2-5AP was lower than that of the pSTM2 in fPBMCs (Fig. 4C).

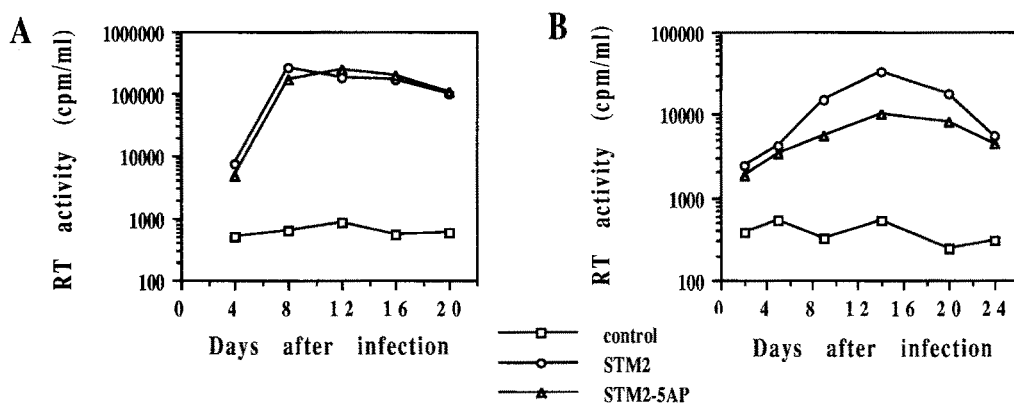
The culture supernatant of MYA-1 cells infected with the virus from pSTM2-5AP at 19 days after infection (Fig. 4A) was transferred onto MYA-1 cells and fPBMCs, and the virus production was monitored by the RT activity assay (Fig. 5). In MYA-1 cells (Fig. 5A), the growth of the virus from pSTM2-5AP was similar to that of the virus from pSTM2. On the other hand, in fPBMCs (Fig. 5B), the growth rate of the mutant was lower than that of the virus from pSTM2.

#### *PCR and sequence analyses*

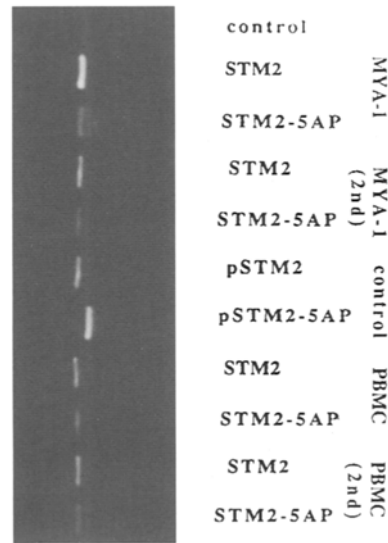
To determine the stability of the insertion of the multiple AP-1 binding sites during the infection experiments (Fig. 4A and C, Fig. 5), the LTRs in the MYA-1 cells and



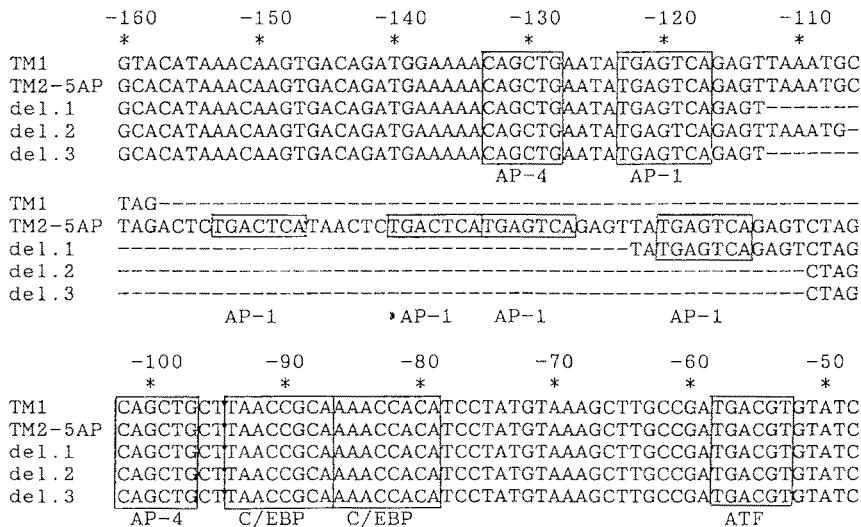
**Fig. 4.** Replication kinetics of the FIVs derived from the infectious molecular clone (pSTM2) (○) and its mutant (pSTM2-5AP) (▲). MYA-1 (A) and FeL-039 (B) cells, and fPBMCs (C) were infected with the same RT counts of FIV derived from the culture supernatant of CRFK cells transfected with each of the infectious molecular clones. These results are representative of several independent experiments



**Fig. 5.** Replication kinetics of the FIVs derived from the culture supernatants of MYA-1 cells at 19 days after infection indicated in Fig. 4A. MYA-1 cells (A) and fPBMCs (B) were infected with the same RT counts of the respective FIVs. These results are representative of several independent experiments



**Fig. 6.** The LTRs of the FIVs were amplified by PCR from extrachromosomal DNAs prepared from MYA-1 cells (*MYA-1*) and fPBMCs (*PBMC*) infected with wild-type (*pSTM2*) or mutant (*pSTM2-5AP*) viruses. The LTRs were amplified from the MYA-1 cells (Fig. 4A) and fPBMCs (Fig. 4C) collected at 29 and 24 days after infection, respectively, and were shown as MYA-1 and PBMC. The LTRs were also amplified from the MYA-1 cells (Fig. 5A) and fPBMCs (Fig. 5C) harvested at 20 and 24 days after infection, respectively. These cells had been infected with the FIVs derived from the culture supernatants of MYA-1 cells at 19 days after infection and were shown as *MYA-1 (2nd)* and *PBMC (2nd)*, respectively. Plasmid DNAs of *pSTM2* and *pSTM2-5AP* were amplified and used as plasmid controls. The left band is a blank control. The PCR products were run through a 1.5% agarose gel and stained with ethidium bromide



**Fig. 7.** Partial nucleotide sequence of the U3 region of the plasmids *pSPTM1* (*TM1*), *pSPTM2-5AP* (*TM2-5AP*), and PCR products of LTR (*del. 1-3*) amplified from DNAs of MYA-1 cells (Figs. 4A and 5A) infected with *pSTM2-5AP* after final samplings. The recognition sequences of enhancer and promoter proteins are boxed: *AP-1* (TGA[C/G]TCA), *AP-4* (CAGCTG), *C/EBP* ([T/A]AACCC[A/G]CA), *ATF* (TGACGT), and TATA box (TATAA)



fPBMCs harvested at final sampling times and were amplified by PCR. As shown in Fig. 6, the length of the mutant LTR was shortened in both MYA-1 cells and fPBMCs harvested at different times. Partial nucleotide sequences of the PCR products obtained from the MYA-1 cells harvested at 29 days after infection (Fig. 4A) and 20 days after infection (Fig. 5) were compared with those of the U3 region of plasmids pSPTM1 (TM1) and pSTM2-5APLTR (TM2-5AP). The same sequence with the pSTM2-5APLTR and three types of deleted sequences (del. 1–3 in Fig. 7) were obtained from the MYA-1 cells infected with the FIV derived from CRFK cells transfected with pSTM2-5AP, and harvested at 29 days after infection (Fig. 4A). The sequence of del. 1 has two AP-1 binding sites and those of del. 2 and del. 3 have only one AP-1 binding site. However, only the sequences of del. 2 and del. 3 were obtained from the MYA-1 cells infected with the FIV mutant derived from the culture supernatants of MYA-1 cells at 19 days after infection, and harvested at 20 days after infection (Fig. 5A), indicating that the insertion of multiple AP-1 binding sites was completely deleted 39 days after infection in the MYA-1 cells.

### Discussion

The LTRs of the lentiviruses contain many binding sites for cellular transcriptional factors. FIV, visna virus and caprine arthritis-encephalitis virus (CAEV) possess putative binding sites of AP-1, AP-4 and C/EBP in the LTRs [7, 8, 20]. Of these, AP-1 and/ or AP-4 binding sites have been shown to be critical for efficient transcription of visna virus [8] and FIV [11, 19, 28]. Although FIV has only one AP-1 binding site in the U3 region of the LTR [14, 26], visna virus and CAEV have multiple AP-1 binding sites in this region [7]. In addition, we found that the promoter activities of visna virus and CAEV LTR are rather higher than that of the FIV LTR in CRFK and SW480 (human colon carcinoma) cells [18]. It has been suggested that the high promoter activities of the visna virus and CAEV are partly due to the multiple copies of the AP-1 binding sites in the LTRs. In this study, by insertion of multiple AP-1 binding sites adjacent to the original AP-1 binding site in the enhancer region of FIV LTR, significant enhancement of promoter activity was observed in CRFK cells. These data suggested that the insertion is effective at least in these cells. However, the activity of the mutant LTR was still lower than that of visna virus LTR. It might be possible that the high promoter activity of visna virus cannot be attributed only to the multiple copies of the AP-1 binding sites. In contrast to the CRFK cells, in fcwf-4 cells no significant enhancement of promoter activity was observed by the insertion, while by deletion of an AP-1 site together with one AP-4 site out of two, the promoter activity was significantly reduced in the cells. The reason for the discrepancy is unknown at present, however it is possible that the promoter activity in fcwf-4 cells is less dependent on the AP-1 site than the deleted AP-4 site.

To examine whether the insertion has positive effect on the replication capability of FIV, we constructed an infectious molecular clone which contained mutated LTRs on both sides of the genome. Firstly, the mutant infectious clone was transfected into CRFK cells, and the transient virus production of the mutant was

compared with that of both the wild-type and the AP-1 deleted mutant. It was expected that the virus production would correlate with the results of the CAT assay. However, no significant difference was observed among these infectious clones. We previously demonstrated the presence of suppressor gene-like activity of FIV which was similar to the *nef* gene activity of primate lentiviruses and reduced the promoter activity of the LTR [19]. It is possible that the suppressor gene-like activity abrogated the enhancement and reduction of promoter activity caused by the insertion and deletion, respectively.

Next, the virus production of the mutant infectious clone was compared with that of the wild-type in feline T lymphocytes. The growth of the mutant virus was delayed in MYA-1 cells, FeL-039 cells and fPBMCs. In addition, when the mutated virus harvested from MYA-1 cells at 19 days after infection (Fig. 4) was transferred onto normal MYA-1 and fPBMCs, the growth of the mutant virus had changed to the level of the wild-type FIV in the MYA-1 cells (Fig. 5A). PCR analysis to amplify the LTR in the cultures revealed that the length of the LTR was shortened to be similar to that of the wild-type virus. Sequence analysis revealed that the insertion of the multiple AP-1 binding sites was completely deleted. These data indicate that the multiple AP-1 binding sites introduced into the LTR have a negative effect on the viral growth activity in feline T lymphocytes, and deletions had occurred by 19 days. Previously we also reported that the AP-1-deleted mutant grew as well as the wild-type in MYA-1 and FeL-039 cells [19] and primary fPBMCs [17], and concluded that the AP-1 site is not required for the replication of FIV in feline T lymphocytes.

The mechanism of the delay of the viral growth of the AP-1 inserted mutant in feline T lymphocytes is unclear at present. However, since the AP-1 binding site (TGA[G/C]TCA) contains palindromic sequence [4], it is possible that multiple AP-1 binding sites introduced in the LTR readily form secondary structures at mRNA level and inhibit reverse transcription from full length genomic RNA to complementary DNA in feline T lymphocytes. Visna virus and CAEV have multiple AP-1 binding sites, however most of the AP-1 sites in both viruses are slightly different from the consensus sequence and are present dispersedly [7, 8]. Therefore it is unlikely that the mRNAs of the U3 region of these viruses form strong secondary structures. Nevertheless, the present study together with the previous studies [17, 18] demonstrates that there is no linear relationship between the promoter activity of the virus determined by the CAT assay and the viral replication capability, therefore to understand the significance of the enhancer and promoter binding sites regarding the virus life cycle, it is necessary to construct infectious clones which lack other binding sites and to compare the growth rates of the mutant viruses.

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