Feline immunodeficiency virus can infect a human cell line (MOLT-4) but establishes a state of latency in the cells

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Infectivity of feline immunodeficiency virus (FIV) in feline and human lymphoblastoid cell lines was examined using homogeneous populations of FIV derived from infectious molecular clones of strains TM2 and Petaluma, and two recombinant chimeric clones carrying gag, pol, vif and ORF-A from the heterologous virus. FIV from the clones with the env region of the Petaluma strain was shown to infect and establish provirus in a human lymphoid cell line (MOLT-4), although the FIV-infected cells did not produce any infectious viruses. By treatment of the infected MOLT-4 cells with a phorbol ester, infectious virus was rescued. To examine which stage of the life-cycle of FIV is blocked in these cells, we analysed transcription of FIV-14 in the cells by RT-PCR. FIV-specific RNA expression could not be detected. These results strongly suggest that latency of the virus in MOLT-4 cells is due to a failure in transcription.

Introduction

Feline immunodeficiency virus (FIV) is a member of the genus Lentivirus of the family Retroviridae. FIV causes persistent infection and induces an immunodeficiency-like disease in cats (Pedersen et al., 1987; Yamamoto et al., 1988, 1989; Ishida et al., 1988, 1989). From its considerable biological and pathogenic similarities to human immunodeficiency virus, FIV infection is regarded as a useful small animal model for human AIDS.

FIV can infect various feline cells in vivo and in vitro, including feline T lymphocytes (Pedersen et al., 1987; Yamamoto et al., 1988; Miyazawa et al., 1989a; Kawaguchi et al., 1991), macrophages (Brunner & Pedersen, 1989) and brain cells (Dow et al., 1990; Kawaguchi et al., 1992a). In addition, biological differences among FIV isolates have been reported. FIV Petaluma strain can infect both a feline CD4 (fCD4)-positive T lymphoblastoid cell line (MYA-1; Miyazawa et al., 1989a) and a fCD4-negative T lymphoblastoid cell line (FL74; Theilen et al., 1969), whereas FIV TM1 and TM2 strains can infect MYA-1 cells but not FL74 cells (Yamamoto et al., 1988; Miyazawa et al., 1989b). FIV-14, originating from an infectious molecular clone of FIV Petaluma strain, can productively infect Crandell feline kidney (CRFK) cells (Olmsted et al., 1989; Phillips et al., 1990), whereas other clones, originating from FIV PPR, TM1 and TM2 strains, cannot infect these cells (Phillips et al., 1990; Miyazawa et al., 1991; Maki et al., 1992). Recently, we showed that the env region of the Petaluma strain determined virus infectivity for CRFK cells (Kohmoto et al., 1994). These data indicate that FIV Petaluma may have a wider host cell range.

Although lentiviruses are considered to be highly species-specific pathogens (Yamamoto et al., 1988), FIV Petaluma was able to form multinucleated giant cells after cocultivation of FIV Petaluma-producing feline T cells (3201) with various human cell lines (Tochikura et al., 1993). Furthermore, integrated FIV provirus DNA was detected in a human lymphoid cell line (MOLT-4) when cocultured with FIV-infected 3201 cells, but the FIV genome-carrying MOLT-4 cells did not produce any infectious virus (Tochikura et al., 1993). These observations suggest the possibility that the FIV Petaluma strain can infect MOLT-4 cells and form provirus DNA following cocultivation with FIV-infected 3201 cells. It remains

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unclear whether other strains, such as TM2, or cell-free FIV can infect MOLT-4 cells, although Tochikura et al. (1993) reported that concentrated cell-free FIV Petaluma induced syncytia in a human lymphoma cell line (MT-2).

The purpose of the present study was to clarify the replication potential of FIV in feline and human cell lines using recombinant chimeric viruses and to determine which stage of the life-cycle of FIV is critically restricted in the cells. We report here that the env region of the Petaluma strain determines the infectivity of cell-free virus for MOLT-4 cells but that virus transcription was restricted.

**Methods**

**Cell cultures and viruses.** MYA-1 cells, which are highly sensitive to FIV (Miyazawa et al., 1989a), were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), antibiotics, 50 μM 2-mercaptoethanol, 2 μg/ml polybrene and 100 units/ml of recombinant human interleukin 2. FL74 cells, which produce feline leukaemia virus (FeLV) (Theilen et al., 1969), a human T lymphoma cell line (MT-4), which produces human T cell leukaemia virus (Miyoshi et al., 1982), and MOLT-4 cells (ATCC CRL 1582) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and antibiotics. These cell lines were passaged every 2 days and maintained at 37 °C in a humidified atmosphere of 5% CO₂. CRFK cells were maintained in Dulbecco's modified Eagle's MEM with 10% FCS and antibiotics. Infectious molecular clones of FIV Petaluma (pFIV-14; Olmsted et al., 1989) and TM2 (pTM219; Maki et al., 1992), and two recombinant chimeric clones (pC1 and pC11; Kohmoto et al., 1994) were used. pC1 was constructed from pTM219 and contained the gag, pol, vif and ORF-A regions of pFIV-14; pC11 was constructed from pFIV-14 and contained the same regions of pTM219 (Fig. 1). To obtain stock viruses, the culture supernatants from MYA-1 cells transfected with pTM219, pFIV-14, pC1 and pC11 were passed through 0.45 μm Millipore membranes after DNase treatment and removal of the cells. The stock viruses were designated TM219, FIV-14, C1 and C11, respectively.

Fig. 1. Oligonucleotide primers and restriction sites of the parental and chimeric infectious molecular clones. A schematic representation of the FIV genome is shown at the top. Short arrows indicate oligonucleotide primers. Primers TG-1, TG-4, PG-1 and PG-4 were used for amplification of FIV-specific DNA. Primers S-1, A-2, A-3, S-4 and A-5 were used for FIV-specific transcripts. The exact positions of these oligonucleotides are given in Methods. Structures of recombinant molecular clones are shown at the bottom.
Reverse transcriptase (RT) assays. Virion-associated RT activities of the culture supernatants were measured as described previously (Willey et al., 1988). For quantification, spots on DE81 paper were cut out and RT activity was recorded by scintillation counting.

Indirect immunofluorescence (IF) assays. For detection of FIV antigens in cell cultures, an IF assay was performed as described previously (Miyazawa et al., 1989).

Nucleic acid extraction. For detection of provirus DNA, total cellular DNA was extracted from 2 x 10^6 MYA-1, FL74, MOLT-4 or MT-4 cells infected with TM219, C1, C11 or TM219 using QiAamp blood kit (Qagen). Total cellular RNA was extracted from 2 x 10^6 CRFK and MOLT-4 cells infected with FIV-14 by the guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987).

Synthetic oligonucleotide primers. Thirteen amplification primers were used in this study. Their sequences and exact positions in the pFIV-14 or pTM219 genomes were as follows: PG-1, CTAGGAGGTTAGGAAATCTATG (nucleotides (nt) 1120 to 1145 of the clone FIV-14); PG-4, CTGGTGAAGCTATTTGTAAACAG (nt 1681 to 1675); TG-1, CTAGGAGGTTAGGAAATCTATG (nt 1126 to 1151 of the clone TM219); TG-4, CTGGTGAAGCTATTTGTAAACAG (nt 1681 to 1675); S-1, GTGAAAGGGACTGACGTTCA (nt 491 to 510 of the clone FIV-14); A-2, GTTCCTGATGATACCCG (nt 794 to 775); A-3, CCGGAGGTTAGGAAATCTATG (nt 5332 to 5313); S-4, GGTCAAGGAGCATTAA (nt 6352 to 6371); A-5, AGTCCTGCTAGGATCTTCT (nt 9040 to 9021). PG-1/PD-4 and TG-1/TG-4 are cDNA-based primer pairs. Primer pair PG-1/PD-4 was used to amplify the FIV-14 and C1 genomes and primer pair TG-1/TG-4 was used to amplify TM219 and C11 genomes. Amplification primers S-1, A-2, A-3, S-4 and A-5 were used for FIV-specific transcripts. The locations of these nine primers are indicated in Fig. 1. CF1 (TATCGACCCCTGTCAAAATATG) and CF2 (TATCGCGGGGTCAGGTAC), which are cat-specific primers that amplify a region of the LTR of endogenous FeLV CF-14, and βG1 (CTTTTTCTCTCTACTCAA) and βG2 (CTCTACTCTCTACTGAG), which are human-specific primers that amplify a region of human β-globin, were used as described previously (Simon et al., 1994).

cDNA synthesis and RT-PCR. To detect FIV-specific transcripts in FIV-14-infected MOLT-4 cells, RT-PCR analysis was performed as follows: 1 µg of DNase-treated RNA in water was heated for 10 min at 70 °C, placed on ice and first-strand cDNA synthesis was carried out in a total volume of 20 µl containing 25 mM-KCl, 50 mM-Tris-HCl pH 8.3, 5 mM-MgCl₂, 0.01 M-DTT, 10 mM of each dNTP, 10 units of RNasin (Toyobo), 10 units of Moloney murine leukemia virus RT (Gibco-BRL) and 50 pmol of the antisense oligonucleotide primer. The reaction mixture was incubated at 45 °C for 2 h. PCR amplification of single-strand DNA was carried out after addition of 50 pmol of the sense oligonucleotide primer and 2.5 units of Taq DNA polymerase (Saki et al., 1988; Perkin-Elmer). The reaction mixture was overlaid with one drop of mineral oil (Sigma) and incubated at 94 °C, 55 °C and 72 °C for 1, 2 and 2 min, respectively. This cycle was repeated 30 times in a Perkin-Elmer Cetus DNA Thermal Cycler P2000.

Analysis of PCR products. PCR products amplified with primer pairs S-4/A-5, S-1/A-3 and S-1/A-2 were analysed on 5% polyacrylamide gels and visualized by UV fluorescence after staining with ethidium bromide. To denature DNA before blotting, gels were soaked in 0.5 M-NaOH and 1.5 M-NaCl for 10 min, washed twice with water and neutralized with 2.0 M-Tris-HCl pH 7.4 and 1.5 M-NaCl for 10 min. The DNA was transferred to nylon membrane by electrophoretic transfer at 35 mA for 16 h in 2 x TBE buffer (0.1 M-Tris-HCl pH 8.3, 0.1 M-boric acid, 2.0 mM-EDTA) at 4 °C. Hybrizations were performed overnight with 32P-labelled probes. The location of the hybridization probes were: A, nt 110 to 3132; B, nt 3132 to 9229. Probes A and B were used for primer pairs S-1/A-3 and S-1/A-2, and for S-4/A-5, respectively.

To detect the PCR products amplified with primer pairs CF1/CF2 and βG1/βG2, PG-1/PD-4 and TG-1/TG-4, DNAs were analysed on 1.8% horizontal agarose gels. DNA was visualized by UV fluorescence after staining with ethidium bromide.

Results

PCR amplification using species-specific primers

Total cellular DNAs were extracted from MYA-1, FL74, MOLT-4 and MT-4 cells and amplified by PCR using species-specific primers CF1/CF2 and βG1/βG2 (Fig. 2a). There was no contamination between cell lines of different species.

Cellular susceptibility to FIV entry

TM219, FIV-14, C1 and C11 were adjusted to similar levels using RT activity (1 x 10^6 c.p.m./ml) and inoculated onto 1 x 10^6 MYA-1, FL74, MT-4 and MOLT-4 cells. The cells were incubated at 37 °C for 1 h and washed three times with RPMI-1640 medium. The genomic DNAs of the infected cells were extracted at 7 days after inoculation. To detect FIV-specific DNA, PCR using primer pairs PG-1/PD-4 and TG-1/TG-4 was performed. As shown in Fig. 2(b), TM219 and C1 gave FIV-specific DNA only in MYA-1 cells, whereas virus DNA of FIV-14 and C11 was detected in FL74 and MOLT-4 cells as well as MYA-1 cells. FIV-specific DNA was not detected in MT-4 cells infected with any of the FIVs. These results indicate that the env region of the Petaluma strain determined virus infectivity for FL74 and MOLT-4 cells and that an infectious molecular clone of FIV with the env region of the Petaluma strain can form provirus in MOLT-4 cells by cell-free infection.

Apparent lack of FIV antigens in FIV-14- or C11-infected MOLT-4 cells

We next investigated whether FIV-specific antigens could be detected in FIV-infected cell lines by IF assay. Cells at 2 weeks after inoculation were examined using FIV antibody-positive fetal serum. As shown in Fig. 2(c), FIV antigens were detected in FIV-14- and C11-infected FL74 cells and in all infected MYA-1 cells. However, no FIV antigen was detected in TM219- or C1-infected FL74 cells, or in any infected MOLT-4 cells. Furthermore, FIV-specific antigen was not detected in MT-4 cells infected with any FIVs (data not shown). In addition, no infectious virus could be recovered from FIV-infected MOLT-4 cells (data not shown). These results suggested that FIV-14- and C11-infected MOLT-4 cells...
Fig. 2. Cellular susceptibility to FIV. (a) PCR amplification using species-specific primers CF1/CF2 (endogenous FeLV) and βG1/βG2 (human β-globin). (b) Detection of FIV provirus DNA. FIV gag gene sequence was amplified from total cellular DNA prepared from FIV-infected MYA-1, FL74, MOLT-4 and MT-4 cells. Lanes 1 and 4, mock-infected cells; lane 2, TM219-infected cells; lane 3, C11-infected cells; lane 5, FIV-14-infected cells; lane 6, C1-infected cells. FIV-specific DNA was amplified by primer pairs TG-1/TG-4 (lanes 1, 2 and 3) and PG-1/PG-4 (lanes 4, 5 and 6). (c) Detection of FIV-specific antigens. The FIV-infected MYA-1, FL74 and MOLT-4 cells at 2 weeks after inoculation were examined by IF assay using FIV antibody-positive feline serum.

Induction of FIV by treatment of FIV-14-infected MOLT-4 cells with a phorbol ester

In FIV, it has been reported that there are many putative protein-binding sites, such as those for AP-1, AP-4, C/EBP and ATF, in the U3 region of the LTR (Kawaguchi et al., 1992b). The putative AP-1 binding site in the FIV LTR is important for responsiveness to stimulation by a phorbol ester (phorbol 12-myristate 13-acetate) and phytohaemagglutin through protein kinase C (Sparger et al., 1992). These data suggest that the putative AP-1 site in the FIV LTR might be activated by phorbol ester treatment and increase virus transcription.

We examined whether infectious FIV could be rescued from FIV-infected MOLT-4 cells by stimulation with a phorbol ester. TM219, FIV-14, C1 and C11 derived from MYA-1 cells were adjusted to similar levels using RT activity (1.5 x 10^6 c.p.m./ml) and inoculated onto 1 x 10^6 MOLT-4...
Fig. 3. Induction of FIV by treatment of FIV-infected cells with TPA. (a) Kinetics of RT activities in FIV-infected MOLT-4 cells. The cells were maintained for 12 days and treated with TPA. The culture supernatants were sequentially analysed for RT activity. These results are representative of two independent experiments. (b) Detection of FIV-specific antigens in FIV-infected MOLT-4 cells at 24 h after TPA stimulation, by IF assay using FIV antibody-positive feline serum. (c) Detection of FIV-specific antigens in MYA-1 cells. Cells inoculated with culture fluids from FIV-infected MOLT-4 cells at 24 h after TPA stimulation were maintained for 7 days. FIV antigens were detected by IF using FIV antibody-positive feline serum.
cells. The culture supernatants were sequentially analysed for RT activity. Twelve days after infection, the FIV-infected cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) at a final concentration of 10 ng/ml for 24 h. As shown in Fig. 3 (a), RT production was transiently raised after treatment with TPA in FIV-14- and C11-infected MOLT-4 cells. When the FIV-infected MOLT-4 cells were examined by IF assay, FIV-specific antigens were detected in the FIV-14- and C11-infected MOLT-4 cells at 24 h after TPA stimulation (Fig. 3 b). In addition, when the culture supernatants from FIV-infected MOLT-4 cells at 24 h after TPA stimulation were filtered and inoculated onto uninfected MYA-1 cells, FIV-specific antigens were detected at 7 days with supernatants from FIV-14- and C11-infected MOLT-4 cells; no antigens were detected using supernatant from TM219- or C1-infected MOLT-4 cells (Fig. 3 c). The same result was obtained using FIV-14-infected MOLT-4 cells 2 months after infection (data not shown). These observations demonstrate that virus could be rescued from the FIV-14- and C11-infected MOLT-4 cells by TPA stimulation and that the recovered virus can infect MYA-1 cells.

**Analysis of transcription patterns of FIV in FIV-14-infected MOLT-4 cells**

Next, to examine which stage of the virus life-cycle was blocked, we analysed the transcription patterns of FIV-14 in untreated and TPA-treated MOLT-4 cells. The splice sites in the FIV-14 genome were predicted from the transcription pattern of several FIV mRNAs which has been reported previously (Phillips et al., 1992; Tomonaga et al., 1992, 1993). RT-PCR was carried out using primer pairs of S-1/A-2, S-1/A-3 and S-4/A-5. The locations of the primers detecting non-spliced, singly spliced and multiply spliced mRNAs are shown in Fig. 1. Upon hybridization, PCR products from the CRFK cells, which were productively infected with FIV-14, yielded all expected bands (Fig. 4 a-c, lane 1). In the FIV-14-infected MOLT-4 cells, multiply spliced and singly spliced mRNAs were not detected (Fig. 4 a, b, lane 2). Although multiply spliced mRNA encoding the Rev protein was not detected, a faint band of non-spliced mRNA was present (Fig. 4 c, lane 2). Since the Rev protein is required for the expression of non-spliced and singly spliced mRNAs (Kiyomasu et al., 1991; Phillips et al., 1992), it suggests either that the multiply spliced mRNA was present at levels below the detectable limit of our assay (Fig. 4 a), or that the faint band observed is an amplified provirus DNA or an amplified genomic RNA of input FIV-14. With TPA treatment of the cells, however, all of the mRNA species were detected as early as 1 day after treatment (Fig. 4, lane 3) and similar transcriptional patterns were obtained at 2 days after treatment (Fig. 4, lane 4).

These results indicated that FIV in infected MOLT-4 cells was in a state of latency with no or extremely low levels of RNA expression but with a fully infectious genome.

**Discussion**

In this study, we demonstrated that the infectivity of cell-free FIV for FL74 and MOLT-4 cells was determined by the env region of the Petaluma strain and that the transcriptional stage of the life-cycle of FIV-14 was blocked in MOLT-4 cells.

We could not rule out the possibility that the infectivity for these cell lines was due to pseudotyping of FIV with endogenous retroviruses in feline cells. However, because TM219 and C1 were not infectious to FL74 and MOLT-4 cells and even FIV-14 and C11 could not infect MT-4 cells, it is not likely that pseudotyping of FIV determines the wider host cell-range of FIV. Recently, we showed that the env region of the Petaluma strain determined virus infectivity for CRFK cells (Kohmoto et al., 1994). Therefore, it is possible that the env gene of FIV-14 virus is responsible for its unique infectivity.

FIV-specific DNA was detected in MOLT-4 cells at 2 months after infection (Fig. 2 c), suggesting the presence of stably integrated provirus in the cells. From this result, the stages of virus penetration, reverse transcription and provirus integration of FIV-14 are considered to be complete in MOLT-4 cells. The activity of the FIV LTR and virus production in FIV-transfected human (HeLa and SW480) and simian (Vero and COS) cells are much lower than in CRFK cells (Miyazawa et al., 1992). Furthermore, in these primate cell lines, the FIV...
Rev protein is much less functional than in feline cell lines (Tomonaga et al., 1994). Moreover, efficient replication of FIV rev mutant virus was observed when only Rev was supplied (Kiyomasa et al., 1991; Tomonaga et al., 1994). Therefore, we hypothesized that FIV expression in MOLT-4 cells was controlled at one or two distinct stages: either a transcriptional stage, due to the low promoter activity of the FIV LTR; or a post-transcriptional stage, due to the deficiency of FIV Rev function. Transcriptional analysis revealed that the FIV genome-carrying MOLT-4 cells were in a state of latency with no or extremely low levels of RNA expression (Fig. 4). In addition, the FIV-infected MOLT-4 cells produced infectious FIV and expressed all classes of mRNA species after TPA treatment. These results indicate that the transcriptional stage determines latency.

In conclusion, we have shown here that cell-free FIV-14 can infect a human cell line in vitro but that transcription of the virus is incomplete. The latent state established in the human cell line will be of interest and importance for understanding the molecular mechanisms involved in maintenance of latent lentivirus infection. Since it is believed that a better understanding of virus latency may be helpful for understanding virus pathogenesis, continued analysis of virus activation in these cells will be important. Furthermore, we believe that these cells will be useful to examine the effects of cytokines, most of which are derived from humans, on integrated FIV.

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