The lentivirus feline immunodeficiency virus (FIV) infects a broad range of cell types, including CD4+ and CD8+ T lymphocytes, B lymphocytes, and macrophages, and analogous to human immunodeficiency virus (HIV) infection of humans, often results in the progressive loss of CD4+ T cells and the eventual development of immunodeficiency in infected cats (2, 9, 21). HIV infection of T lymphocytes involves attachment of the viral envelope glycoprotein to the specific cellular receptor CD4 (5, 14). However, this is usually not sufficient to confer susceptibility to HIV infection (4, 17), and HIV requires a member of the chemokine receptor family as a coreceptor (6, 7, 11). FIV does not utilize CD4 for entry (12, 18). However, FIV subtype A viruses adapted for growth in the feline CrFK cell line utilize CXCR4 as a receptor (23; B. J. Willett, M. J. Hosie, J. C. Neil, J. D. Turner, and J. A. Hoxie, Letter, Nature 385:587, 1997). Human U87 cells expressing CXCR4 supported the formation of syncytia when infected with FIV Peta-luma and FIV Glasgow-8 (30), but no productive infection was detected. Additionally, recent work indicates that at least some primary FIV isolates use CXCR4 for cell entry (26). In this study, we examined the receptor requirements and viral DNA replication intermediates of FIV. Based on characteristics common to all retroviruses, including genomic organization and the process of reverse transcription (8), we developed a cell entry assay for FIV. The product of reverse transcription that is preferentially integrated into the host chromosome to establish a productive infection is a linear DNA molecule that begins with a 5’ long terminal repeat (LTR) and ends with a 3’ LTR (3). However, two circular forms of unintegrated viral DNA are also found in the nucleus and serve as markers of a DNA genome intermediates. Our findings extend the role of CXCR4 as a primary receptor for CD4-independent cell entry by FIV.

The process of feline immunodeficiency virus (FIV) cell entry was examined using assays for virus replication intermediates. FIV subtype B was found to utilize the chemokine receptor CXCR4, but not CCR5, as a cellular receptor. Zidovudine blocked formation of late viral replication products most effectively, including circular DNA genome intermediates. Our findings extend the role of CXCR4 as a primary receptor for CD4-independent cell entry by FIV.

**FIV 34TF10 entry into CrFK cells.** Infection of CrFK cells with subtype A FIV 34TF10 was monitored over time by PCR amplification of viral genome fragments that represented early, intermediate, and late stages of the reverse transcription process (Table 1 and Fig. 1). The virus stock was generated by transfection of the 34TF10 plasmid into CrFK cells, with the supernatants being combined, filtered (0.45-μm pore-size), aliquoted, and stored at −80°C prior to use at a final dilution of 70 50% tissue culture infective doses. β-actin gene amplification was included as a control for DNA quantitation and PCR efficiency, using primers designed as described previously (27) to amplify from both feline and human DNA. The β-actin PCR products were ~600 bp for human and ~1,000 bp for feline products. PCR (reagents and protocols from Bioline, Reno, Nev.) cycling parameters included denaturation for 3.5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C (or 58°C for the LTR primer set or 50°C for the β-actin primers), and 1 min at 72°C (the final incubation was for 10 min). The PCR product that represented an early product of reverse transcription was the LTR fragment, amplified with the LTR-F and LTR-R primers (Table 1 and Fig. 1). The intermediate product was the LTR-Gag fragment, amplified with the LTR-F and GAG-R primers, and the late product was the circle junction fragment, amplified with the ENV-F and GAG-R primers. These amplifications were carried out using the cell lines, virus stocks, and PCR controls shown in Table 2 as targets.

The sensitivity of each primer set was determined using serial dilutions of p34TF10 and/or a plasmid containing the circle junction fragment. The threshold for detection of the 163-bp LTR product was 100 copies, and the threshold was 10 copies for the 542-bp LTR-gag and 967-bp circle junction products (data not shown). LTR and LTR-gag products were present at 6 h postinoculation (PI) (Fig. 1A) and became progressively stronger during the course of the experiment. The circle junction product was detected at 45 h PI and became stronger with time. Consistent β-actin levels were found at 6, 20, 30, and 45 h PI, with lower levels present at 0 and 70 h. This experiment shows that early and intermediate products are detected soon after infection of CrFK cells with FIV 34TF10 but that circular products are not detected until between 30 and 45 h PI.
Putative FIV circle junction DNA from CrFK cells infected with FIV 34TF10 was PCR amplified, cloned, sequenced, and identified as a one-LTR circle junction (Fig. 1C). A faint product most likely corresponding to a two-LTR circle junction was also detected in the PCR products analyzed. Our results are consistent with studies of HIV type 1 (HIV-1) infection that showed that the two-LTR form is less abundant than the one-LTR form in tissue culture (10, 20).

**TABLE 1. PCR oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Polarity</th>
<th>Sequence (5’ to 3’)</th>
<th>Nucleotide positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTR-F</td>
<td>Plus</td>
<td>GCGCTAGCAGCTGCTTAACCGCAAACAC</td>
<td>108–137</td>
</tr>
<tr>
<td>LTR-R</td>
<td>Minus</td>
<td>GTATCTGTGGAGGCTCAAGGGGAACTC</td>
<td>240–269</td>
</tr>
<tr>
<td>GAG-R</td>
<td>Minus</td>
<td>CGCCCCTGCTCATCCTCCTCATGTGCTGAGATCC</td>
<td>612–646</td>
</tr>
<tr>
<td>ENV-F</td>
<td>Plus</td>
<td>GGCAATGTGGCAGATGCTGGGAAAGGAGGAATGATG</td>
<td>8802–8839</td>
</tr>
<tr>
<td>β-actin-F</td>
<td>Plus</td>
<td>ATGTGGCAAGGCGGCTTTCG</td>
<td>119–137</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>Minus</td>
<td>TTAATGTCAGCGCAGATTTCC</td>
<td>691–711</td>
</tr>
</tbody>
</table>

* Nucleotide positions were based on the FIV Petaluma sequence (bases 1 to 9474) (19).

**FIG. 1.** CrFK cell infection with FIV 34TF10 and detection of viral replication intermediates. (A) CrFK cells were infected with FIV 34TF10 in replicate wells and harvested from one well at 0, 6, 20, 30, 45, and 70 h PI. PCR was performed as described in the text, with FIV 2542-CrFK cellular DNA (FIV+) as the positive control and H2O plus reagents (H2O) as the negative control. The DNA marker is a 100-bp or 1-kb ladder, as indicated. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. (B) Schematic representation of primer positions for derivation of PCR products in FIV-infected cells derived from linear and circular viral DNA is shown. (C) One-LTR circle junction fragment homology (shaded segments) is indicated. As shown at the bottom of panel C, the fragments produced after FIV 34TF10 infection were colinear with that expected from the FIV 34TF10 genome.

**TABLE 2.** Cell lines, viral stocks, and PCR controls utilized in this study

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Viral stocks</th>
<th>PCR controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CrFK</td>
<td>FIV-A from CrFK cells</td>
<td>H2O reagent control</td>
</tr>
<tr>
<td>U87-T4</td>
<td>FIV-B from CrFK cells</td>
<td>34TF10 CrFK DNA</td>
</tr>
<tr>
<td>U87-T4-CXCR5</td>
<td>FIV-C from PBMC</td>
<td>34TF10 plasmid</td>
</tr>
<tr>
<td>U87-T4-CXCR4</td>
<td>FIV 34TF10</td>
<td>Molecular weight marker</td>
</tr>
</tbody>
</table>
Lack of FIV DNA in viral stocks. Detection of LTR products early in infection could be due to new synthesis of viral DNA upon cell entry or to incomplete viral transcripts carried in the FIV particle (16). The DNase treatment we employed would eliminate DNA present in the supernatant but not from within the viral particle. We therefore examined viral inocula for the presence of viral DNA. Peripheral blood mononuclear cell (PBMC) viral stocks were derived by coculture of PBMC from uninfected cats and specific-pathogen-free capture cats infected with FIV field isolates 2546 (FIV-A) or 2542 (FIV-B) (1). CrFK-grown subtype A and B viruses were derived from chronically infected CrFK cells infected by coculture with supernatant from FIV-positive PBMC cultures. An equivalent amount of virus, quantitated using p24 antigen, was used for each infection. PCR on the viral inoculum did not result in detectable product (Fig. 2A); hence, the PCR signal detected in the entry assay could not be attributed to DNA present in the viral inoculum. Consistent with our results, less than 1% of the HIV-1 (−) single-stranded DNA found in infected cells can be attributed to DNA carried into the cell inside the viral particle (25).

FIG. 2. Lack of FIV DNA in viral stocks and impact of AZT on the production of viral DNA intermediates. (A) Viral inocula were examined for the presence of viral DNA by PCR. Viral stocks corresponded to FIV-A grown in CrFK cells (AC), FIV-B grown in CrFK cells (BC), FIV-B grown in PBMC (BP), FIV-C grown in PBMC (CP), and FIV 34TF10 (34) grown in CrFK cells. FIV+ corresponds to a positive control (FIV-infected CrFK DNA). (B) CrFK cells were infected with FIV 34TF10 in replicate wells, differing only by the addition of AZT-1MP. Cells were harvested at 0, 24, 72, and 144 h and immediately lysed and stored for subsequent DNA extraction. PCR was performed using 50 ng of DNA as a template. PCRs were separated by agarose gel electrophoresis and visualized by ethidium bromide staining (10 μl per sample). The marker (MW) for the LTR and LTR-gag fragments was a 100-bp ladder and for the circle and β-actin fragments was a 1-kb ladder. Controls were H2O plus reagents (H2O), CrFK DNA (CrFK), FIV 34TF10-infected CRFK DNA (FIV+), and p34TF10.
FIG. 3. AZT can block circle formation in CrFK and U87-T4-CXCR4 cells infected with CrFK-derived FIV-A or FIV-B. FIV subtype A or FIV subtype B was used to infect CrFK, U87-T4, U87-T4-CCR5, and U87-T4-CXCR4 cell lines in replicate wells differing only by the addition of AZT. Cells were harvested, and viral sequences were PCR amplified and visualized as described in the legend to Fig. 2. The figure depicts the LTR PCR (A), the LTR-Gag PCR (B), the circle fragment PCR (C), and the β-actin PCR (D). The PCR for each fragment was performed concurrently for all four cell lines. Four controls were included for each amplification and were run in lane 2 (PCR CNTL) of the four gels in each panel, with the first gel of each panel containing the H2O control, the second gel containing the CrFK control, the third gel containing the 34TF10 CrFK control, and the fourth gel containing the 34TF10 plasmid. AC, FIV-A grown in CrFK cells; BC, FIV-B grown in CrFK cells.
AZT blocked circle formation in CrFK cells infected with 34TF10. We monitored the production of viral DNA following FIV 34TF10 infection of CrFK cells in the presence of zidovudine (AZT-1MP) (Sigma; A6806; 10 μg/ml), a nucleoside analogue inhibitor of reverse transcription, or Dulbecco’s modified Eagle’s medium alone as a control (Fig. 2B). For the experiments shown in Fig. 2B and 3, cells were seeded with 2× AZT-1MP or Dulbecco’s modified Eagle’s medium alone and incubated for 1 h to allow cells to convert AZT to the triphosphate form. Prior to infection, viral stocks were DNase I treated and filtered (15). The LTR fragment was present at 0 h PI and the signal increased with time for all samples (Fig. 2B). The intensity of the LTR-gag product also increased over time in untreated cells but was less intense, especially at later time.
points, for the AZT group. Circle junction products were detected by 72 h PI in the untreated sample but were not detected for the AZT-treated group up to 144 h PI. Our results were consistent with previous studies showing that inhibitors of reverse transcription are most effective against long products of reverse transcription (24, 25, 29, 31).

FIV-B utilizes CXCR4 for entry and AZT blocks circle formation. We next sought to determine whether CrFK cell line-adapted FIV with envelope sequence subtype B, as with subtype A (23, 30; Willett et al., letter), used CXCR4 for viral entry (Fig. 3). We infected CrFK, U87-T4, U87-T4-CCR5, and U87-T4-CXCR4 cell lines with FIV subtype A or B virus and monitored the production of viral DNA in replicate wells in the presence of AZT or medium alone. Chemokine receptor expression was verified by staining with fluorescently labeled antibodies specific for CXCR4 (12G5 CXCR4-PE) or CCR5 (2D7 CCR5-FITC) prior to infection (Pharmingen, San Diego, Calif.) (27). Fluorescein isothiocyanate- and phycoerythrin-mouse immunoglobulin G2a kappa-isotype control antibodies G155-178-FITC and G155-178-PE (PharMingen) were used as controls for nonspecific binding. Cells were then analyzed by cytfluorometry using a FACScan flow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems). To show that the CCR5-expressing cell line was functional and

FIG. 4. FIV antigen production corresponds to circle formation during FIV infection. Lysed cells and supernatants were examined at days 10 and 17 PI. (A) Circle and β-actin fragment PCR results from days 10 and 17 PI. The positive control was 34TF10 CrFK DNA and the negative control was an H$_2$O reagent control. The PCR controls were run in lane 2 (PCR CNTL), with the H$_2$O reagent control on the top gel for each fragment and the positive control on the bottom gel for each fragment. The DNA marker was the 1-kb ladder. (B) Results from the enzyme-linked immunosorbent assays for FIV antigen. T4, U87-T4 cells; R5, U87-T4-CCR5 cells; X4, U87-T4-CXCR4 cells. Virus designations are defined in the legend to Fig. 2.
competent for viral infection, we infected the U87-T4-CCR5 cell line with simian immunodeficiency virus isolate 239 (SIV-239), and the resulting supernatant tested positive for SIV antigen (p27) at days 10 and 14 PI (data not shown). Cells were harvested at 0, 24, 72, and 144 h PI and immediately lysed and stored for subsequent DNA extraction and PCR amplification. Cells were harvested for the 0-h time point and lysed after exposure to virus within 10 min. However, this brief exposure of cells to virus resulted in the production of reverse transcription products detectable at 0 h PI in some samples. The LTR (Fig. 3A) and LTR-Gag (Fig. 3B) PCR products were detected for both of the viral subtypes in CrFK and U87-T4-CXCR4 cells but not in the U87-T4 or U87-T4-CCR5 cells. AZT partially inhibited formation of both the LTR and LTR-Gag fragments in CrFK and U87-T4-CXCR4 cells. Differences were also evident between the susceptible cell lines, as products were present at 0 h in CrFK but not in U87-T4-CXCR4 cells. The PCR signal was stronger for the samples without AZT than for those incubated with AZT for both cell lines and both viral subtypes. The signal for CrFK cells increased with time, while infected U87-T4-CXCR4 cells showed a strong and continuing signal beginning at 24 h PI. Circle formation was detected only in the absence of AZT and was detected earlier in the U87-T4-CXCR4 cells than in the CrFK cells for both the FIV-A and FIV-B infections (Fig. 3C). The signal for the β-actin fragment was consistent overall (Fig. 3D). This experiment demonstrates that early and intermediate viral DNA products are formed in permissive cells even in the presence of AZT but that late viral DNA products are only formed at appreciable levels in permissive cells in the absence of AZT.

**FIV-B antigen production in CXCR4-transfected cells.** We observed the production of syncytia by day 12 of the FIV-B 2542 infection (~10/well) in U87-T4-CXCR4 cells, massive cytopathic effects and many large floating syncytia were present on day 13 (~90/well), and the cultures were terminated due to cytopathic effects on day 14 (data not shown). This demonstrated that FIV subtype B can utilize CXCR4 for cell entry and demonstrated productive infection of FIV-B in U87-T4-CXCR4 cells.

**FIV antigen production corresponds to circle formation during FIV infection.** We next determined if productive FIV infection, as indicated by the presence of FIV p24 Gag antigen, corresponded to circle formation during FIV infection (Fig. 4). Viruses were used to infect CrFK, U87-T4, U87-T4-CCR5, and U87-T4-CXCR4 cell lines. Cellular DNA and culture supernatants were harvested on days 10 and 17 PI. The cellular DNA was used for PCR and the culture supernatants were tested for FIV antigen using the FIV PetChek enzyme-linked immunosorbent assay kit (Idexx Laboratories, Westbrook, Maine). The samples positive for p24 antigen were the same ones in which circular viral DNA was detected (Fig. 4). The PBMC-derived subtype B and C FIVs were negative for both viral DNA intermediates and antigen in the cell lines tested. We did observe circle formation or FIV antigen for any of our experiments that used PBMC-derived FIV-B or FIV-C, indicating that the block to replication in cell lines is not overcome by the transfection of CXCR4 alone. Most primary isolates of FIV infect PBMC, but only subsets have been adapted to infect CrFK cells, including the FIV-B PBMC-derived virus used in this study. It is not clear why some primary isolates grown only in PBMC, such as the FIV-C PBMC-derived virus used in this study, cannot efficiently be adapted to grow in CrFK cells, given that CrFK cells have been shown to express CXCR4 mRNA (30) and some primary isolates have been shown to use CXCR4 for entry (26). It may be that some FIV strains use a receptor other than CXCR4, but when they are adapted to grow in CrFK cells, the receptor usage switches to CXCR4 in a manner parallel to HIV adaptation for growth in T-cell lines. All three FIV-derived viruses tested (FIV-A, FIV-B, and 34TF10) were positive for both circle formation and viral antigen when infecting CrFK or U87-T4-CXCR4 cells but not U87-T4 or U87-T4-CCR5 cells. The only exception was that FIV 34TF10 was positive for circle formation in the U87-T4-CXCR4 cells, but viral antigen was not detected (although p24 was detected in other experiments [data not shown]). The presence of HIV-1 circles has been suggested to be a molecular indicator of the disease progression of HIV-1 (13, 32). Hence, the assessment of circle formation as a predictor of FIV disease progression could be evaluated using the procedures described here for future studies.

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