The C/EBP Site in the Feline Immunodeficiency Virus (FIV) Long Terminal Repeat (LTR) Is Necessary for Its Efficient Replication and Is Also Involved in the Inhibition of FIV LTR-Directed Gene Expression by Pseudorabies Virus ICP4

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We investigated effects of site-specific mutation of the putative C/EBP binding site in the feline immunodeficiency virus (FIV) long terminal repeat (LTR) on the basal promoter activity in Crandell feline kidney (CRFK) cells and on replication efficiency in CRFK cells and a T-lymphoblastoid cell line, MYA-1 cells. Mutation of the C/EBP site reduced the basal promoter activity in CRFK cells and prevented efficient FIV replication in both CRFK and MYA-1 cells. Gel-mobility-shift assay using nuclear extracts from CRFK and MYA-1 cells revealed that the nuclear factor(s) actually binds to the C/EBP site, but there was a clear difference in the binding patterns to the C/EBP site between CRFK and MYA-1 cell nuclear proteins. Furthermore, we demonstrated that the C/EBP site is necessary for inhibition of FIV LTR-directed gene expression by pseudorabies virus (PRV) ICP4. The C/EBP site is sufficient to confer inhibitory effect by PRV ICP4 on heterologous promoters. These data suggest that the C/EBP site in the FIV LTR is important for the positive regulation of FIV gene expression and replication and is also required for the negative regulation of FIV gene expression by PRV ICP4. © 1995 Academic Press, Inc.

INTRODUCTION

Feline immunodeficiency virus (FIV) belongs to the genus *Lentivirus* of the family Retroviridae (Miyazawa and Mikami, 1993; Miyazawa *et al.*, 1994; Sparger *et al.*, 1989). FIV shares some biological characteristics with primate lentiviruses such as cell tropisms for T-lymphocytes and macrophages *in vivo* (Miyazawa and Mikami, 1993; Sparger *et al.*, 1989) and association with immunodeficiency-like diseases in cats (Miyazawa and Mikami, 1993; Miyazawa *et al.*, 1994; Sparger *et al.*, 1989). In contrast, genomic organization and functional transcriptional regulatory sequences in the long terminal repeat (LTR) of FIV appear to be similar to nonprimate lentiviruses such as visna virus, caprine arthritis–encephalitis virus and equine infectious anemia virus (EIAV) (Miyazawa *et al.*, 1994; Sparger *et al.*, 1989).

Lentivirus gene expression is governed by cellular factors through transcriptional regulatory sequences in the LTR of the virus and by the viral-encoded regulatory proteins (Cullen and Green, 1989; Narayan and Clements, 1990). We and other groups previously identified the function of the viral-encoded regulatory gene product Rev, which is functionally similar to those of the other lentivi-

ruses and was characterized to control splicing of virus mRNA through a *cis*-acting target sequence (Kiyomasu *et al.*, 1991; Phillips *et al.*, 1992). Recently, our group also identified another regulatory gene, ORF A, which is thought to correspond to Tat of the other lentiviruses; however, the ORF A product could not activate the LTR of FIV, and the ORF A product is critical for efficient viral replication in primary peripheral blood lymphocytes (Tomonaga *et al.*, 1993).

In the U3 of the FIV LTR there are many putative transcriptional regulatory sequences (Miyazawa *et al.*, 1994). We and others identified the regions which are important for viral gene expression (Kawaguchi *et al.*, 1992; Miyazawa *et al.*, 1993; Sparger *et al.*, 1992; Thompson, F. J., *et al.*, 1994). Sparger *et al.* (1992) reported that site-directed mutation of AP-1, AP-4, or ATF site in the FIV LTR resulted in a depression of promoter activity in Crandell feline kidney (CRFK) cells and G355 cells derived from fetal brain tissue. We and Thompson, F. J., *et al.* (1994) indicated that the C/EBP site, in addition to the AP-1, AP-4, and ATF sites, was also critical for the basal promoter activity in *Felis catus* whole fetus-4 (fcwf-4) and CRFK cells by using 5′ sequential deletion mutants of the LTR (Kawaguchi *et al.*, 1992). The AP-1 site has also been reported to be required for the responsiveness to the T cell activation (Sparger *et al.*, 1992) and the activation by c-Fos (Miyazawa *et al.*, 1993). The ATF site and the sequence −63 to −44 (relative to cap site) which contains the ATF site were found to be the targets for c-AMP-induced responses (Sparger *et al.*, 1992) and re-

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sponsiveness to feline herpesvirus type 1 (Kawaguchi et al., 1992), respectively. In contrast, only limited information is available about the C/EBP site. Although 5' sequential deletion including the C/EBP site reduces the promoter activity in CRFK and fcwf-4 cells (Kawaguchi et al., 1992; Thompson, F. J., et al., 1994), the deletion also removes the AP-4 and AP-1 sites; therefore, a specific function of the C/EBP site for viral gene expression and replication is uncertain at present. C/EBP sites are also present in the LTRs of EIAV and the other retroviruses and play important roles in the gene expression of the viruses (Boral et al., 1989; Derse et al., 1993; Fulton et al., 1990; Thornell et al., 1988). In the present study, we examined the effect of site-specific mutation of the C/EBP site in the FIV LTR on the viral gene expression and replication. Results indicated that the C/EBP site is critical not only for FIV LTR-directed gene expression in CRFK cells but also for viral replication in both CRFK and feline T-lymphoblastoid cells. Furthermore, we demonstrated that the C/EBP site is involved in the inhibition of FIV LTR-directed gene expression by pseudorabies virus (PRV) ICP4.

MATERIALS AND METHODS

Cells

CRFK cells (Crandell and Despeaux, 1959) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. MYA-1 cells (Miyazawa et al., 1989b), a feline T-lymphoblastoid cell line, were maintained in RPMI 1640 medium supplemented with 10% FCS, antibiotics, 50 μM 2-mercaptoethanol, 2 $\mu g/ml$ of polybrene, and 100 units/ml of recombinant human interleukin-2.

DNA constructs

pTM1 CAT which contains the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the FIV LTR was described previously (Kawaguchi et al., 1991). pHd CAT, used as a negative control plasmid, was described (Shibata et al., 1990). An effector plasmid (pRVPRIE) expressing PRV ICP4 gene was constructed using pRVSV which contains a strong constitutive promoter cassette of the Rous sarcoma virus LTR as described elsewhere (Kawaguchi et al., 1994; Sakai et al., 1990). To construct linker-scanner mutants of the FIV LTR, two of the 5' deletion mutants (5' −107 and 5' −79) of pTM1 CAT which were previously described (Kawaguchi et al., 1992) were used. Specific fragments of pTM1 CAT were amplified by polymerase chain reaction (PCR), using a range of oligonucleotide primers containing EcoRI linkers at both termini. The amplified fragments were ligated into the EcoRI sites of 5' −107 and 5' −79 to generate linker-scanner mutants [pdEBP CAT and pdAP1

CAT (Fig. 1)] pTM1 LTR and pdEBP LTR were generated from pTM1 CAT and pdEBP CAT by deleting a HindIII fragment which contains the CAT gene and simian virus (SV) 40 small t intron and polyadenylation signal. To construct an internal-deletion mutant of the FIV LTR, a KpnI− HindIII subfragment of pTM1 LTR was cloned into plasmid Bluescript(KS⁺), and then an AflIII−PstI subfragment of pTM1 LTR (the AflIII-cut ends were blunted by treatment with Mung Bean nuclease) was inserted into HindIII and PstI sites of the plasmid (the HindIII-cut ends were blunted by treatment with T4 DNA polymerase) and designated pdATF LTR. pdATF CAT was constructed by cloning a KpnI−XbaI subfragment of pdATF LTR into pHd CAT (Fig. 1). All constructs described above were confirmed by sequencing across the mutated region and/or the amplified regions in each construct by PCR.

To introduce the mutation of the C/EBP site into infectious molecular clones (IMCs) of FIV, we used two IMCs, termed pSTM2 (Miyazawa et al., 1993) and pFIV14 (Olmsted et al., 1989). FIV derived from the pFIV14 is able to infect CRFK cells, whereas FIV derived from pSTM219 is unable to infect CRFK cells (Maki et al., 1992; Olmsted et al., 1989). To construct pCtTM and pdTM, a SacI fragment of pSTM2 which contains the 5' LTR of the clone was subcloned into pUC19, and an AflIII fragment of the plasmid was substituted with an AflIII fragment of pTM1 LTR or pdEBP LTR. These are designated as pTM1 Sc and pdEBP Sc, respectively. Each of the SacI fragments of pTM1 Sc and pdEBP Sc was replaced with the SacI fragment of the pSTM2, and these are designated pCtTM and pdTMD1, respectively. A SphI fragment of pSTM2 which contains the 3' LTR of the clone was subcloned into pUC19 and designated p3'Sph. A BamHI−PstI fragment of pdEBP LTR was substituted with a BamHI−PstI fragment of p3'Sph and designated pdEBP Sph. A SphI fragment of pdEBP Sph was replaced with a SphI fragment of pdTMD1 and designated pdTM. To construct pCt14 and pd14, a NheI−KpnI fragment of pFIV14 was substituted with a NheI−KpnI fragment of pTM1 LTR or pdEBP LTR and designated pCt14env and pd14env, respectively. A SacI−KpnI fragment of pFIV14 was cloned into SacI and KpnI sites of pCt14env or pd14env and designated pCt14D1 and pd14D1, respectively. The SacI fragments of pTM1 Sc and pdEBP Sc were cloned into SacI sites of the pCt14D1 and pd14D1, respectively, and these are designated pCt14 and pd14. The resultant plasmids bear gag, pol, and env regions of pFIV14, and LTRs of pTM1 LTR and pdEBP LTR.

pH1o CAT was constructed by deleting an AvaI fragment of pH1 CAT (Shibata et al., 1990). To construct pH1EBP CAT and pH1dEBP CAT, a NheI−HindIII fragment of pTM1 LTR (relative to the cap site of −106 to −65 containing the C/EBP site) or a NheI−HindIII fragment of pdEBP LTR containing a mutated version of the C/EBP site was cloned into pH1o CAT (Fig. 2). pH1EBP2 CAT and pH1dEBP2 CAT were generated by additional inser-
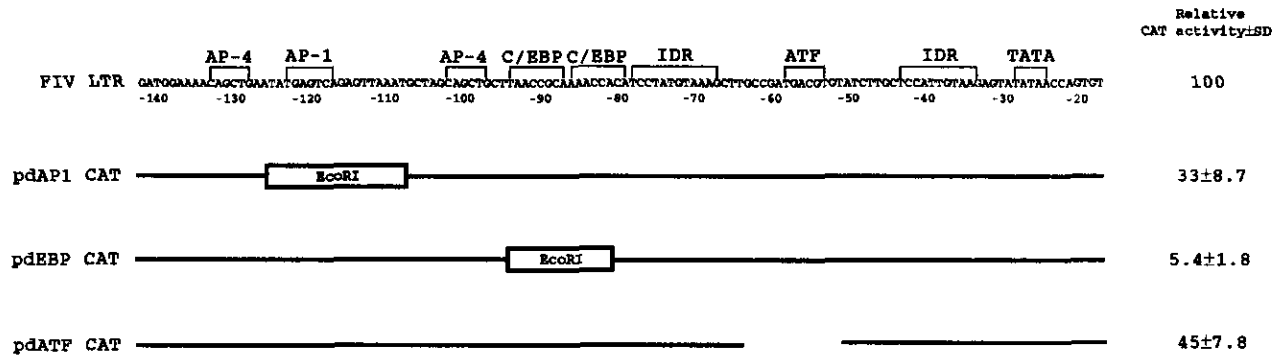
FIG. 1. Construction of specific mutants of the FIV LTR and the promoter activity of the constructs in CRFK cells. The top line shows sequence of the FIV LTR from −142 to −17. The relative positions of the AP-1, AP-4, C/EBP, and ATF sites, the imperfect direct repeat (IDR), and TATA element are indicated. The numbers below the sequence refer to nucleotide positions relative to the cap site. Also shown are the positions of the linker-scanner mutations as well as the endpoints of deletion (pdATF CAT). Boxes indicate the position of the EcoRI linker. Relative CAT activities of the constructs in CRFK cells were obtained from the results of the transfection assays with the constructs and pRVSV shown in Table 1 and the data represent the average results of three independent experiments.

tion of the Nhel−HindIII fragments of pTM1 LTR and pdEBP LTR into pH1EBP CAT and pH1dEBP CAT, respectively (Fig. 2).

DNA transfections

For transfection of plasmid DNAs into CRFK cells, the cells were plated in six-well plastic microplates. Plasmid DNAs were transfected by the calcium phosphate method (Graham and Van der Eb, 1973). At 4 hr after transfection, the cells were washed with DMEM and glycerol-shocked, and then fresh medium was added. Amounts of reporter plasmids transfected are indicated in Tables 1 and 2 and Fig. 2. Effector plasmids were transfected in equimolar amounts (0.9 pmol). The final amount of DNA applied per transfection was adjusted to an equal amount by addition of pUC19.

MYA-1 cells were transfected by electroporation according to the protocol of Barry et al. (1989). Briefly, the cells were washed with phosphate-buffered saline (PBS) and resuspended at a density of 1×10^7 cells per ml in RPMI 1640 [without FCS; 10 mM dextrose; 0.1 mM dithiothreitol (DTT)]. Four hundred microliters of cells in a 0.4-cm cuvette was electroporated with 20 μg of IMC at 300 mV and 960 μFD. At 5 min postelectroporation, the cells were placed into growth media and were cocultivated with normal MYA-1 cells (1.5×10^6).

CAT assay

At 48−72 hr after transfection, cell monolayers were harvested for the CAT assay. Cell extracts were prepared as described previously (Gorman et al., 1982). CAT activity was assayed by the solvent partition method as de-
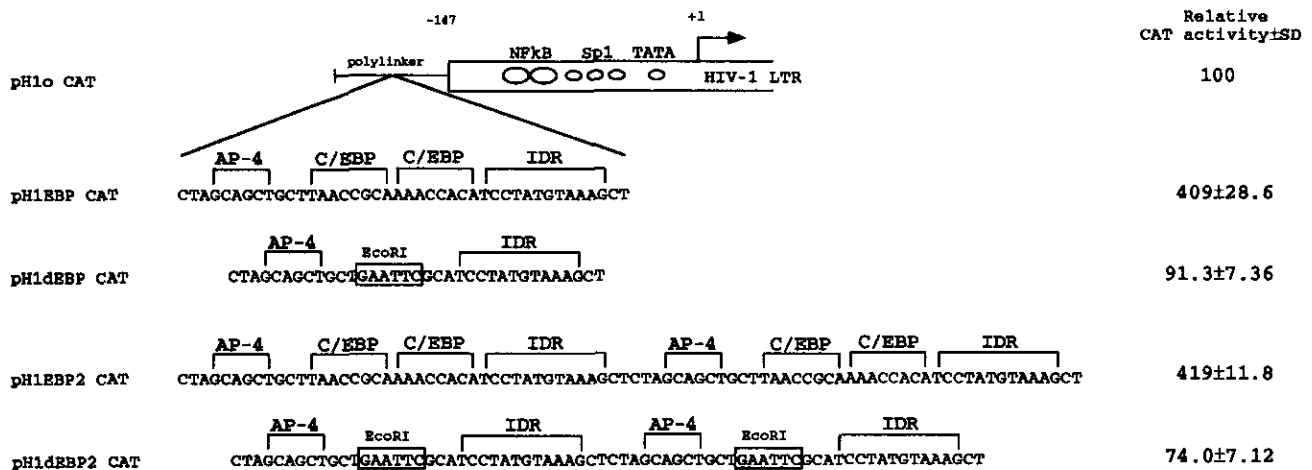

FIG. 2. Construction of heterologous promoter fused to the FIV C/EBP site and the promoter activity of the constructs in CRFK cells. pH1o CAT contains an Aval−NarI fragment of the HIV-1 LTR. pH1EBP CAT contains the region (−106 to −65) of the FIV LTR ligated 5′ to pH1o CAT. pH1dEBP CAT contains the region of the FIV LTR including a mutated version of the C/EBP site. pH1EBP2 CAT was constructed by inserting the region of the FIV LTR upstream of the pH1o CAT in duplicate. pH1dEBP2 CAT was generated by placing the region of the FIV LTR including a mutated version of the C/EBP site upstream of the pH1o CAT in duplicate. Relative CAT activities of the constructs in CRFK cells were obtained from the results of the transfection assays with the constructs (0.5 μg) and the data represent the average results of three independent experiments.

scribed previously (Neumann et al., 1987) and was presented as the net dpm of products formed per hour.

Virus detection

The Mg^{2+}-dependent reverse transcriptase (RT) activities in culture supernatants were assayed as described previously (Ohta et al., 1988). An indirect immunofluorescence (IF) assay was performed to detect FIV antigens in cell cultures as described (Miyazawa et al., 1989a).

Gel-mobility-shift assay

Nuclear extracts from CRFK and MYA-1 cells were prepared as described previously (Schreiber et al., 1989). Briefly, $1-2 \times 10^6$ cells were collected, washed with PBS, and resuspended in 400 μl cold buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF). After the cells were allowed to swell on ice for 15 min, 25 μl of a 10% solution of Nonidet-P40 was added and the tube was vigorously vortexed for 10 sec. After centrifugation, the nuclear pellet was resuspended in 50 μl ice-cold buffer C (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF) and the tube was vigorously rocked at 4° for 15 min on a shaking platform. The nuclear extracts were centrifuged for 5 min at 4° and the supernatant was frozen in aliquots at −80°.

For gel-mobility-shift assays, nuclear extracts were incubated at room temperature for 20 min with binding buffer (0.05 mg/ml poly(dI−dC)poly(dI−dC); 10 mM Tris−HCl, pH 7.5; 50 mM NaCl; 1 mM MgCl$_2$; 0.5 mM DTT; 0.5 mM EDTA; 4% glycerol). After incubation, 0.175 pmol of ^{32}P-labeled oligonucleotide probe was added and the mixture was incubated for an additional 20 min at room temperature. Samples were then applied to a nondenaturing 4% polyacrylamide in 0.5× TBE gel. After electrophoresis, the gel was dried and autoradiographed.

RESULTS

Enhancer activity of the C/EBP site in the FIV LTR

We examined effects of each of the site-specific mutants of the AP-1, ATF, or C/EBP site on the basal promoter activity in CRFK cells. Deletions of the AP-1 or ATF site reduced the LTR-directed CAT activity almost two- to threefold (Fig. 1). In addition, deletion of the C/EBP site dramatically reduced the LTR activity almost 15-fold (Fig. 1). To test the enhancer activity of the C/EBP site more directly, the effect of insertion of the C/EBP site into a heterologous promoter on its promoter activity was examined. When we placed one or two copies of the Nhe I−Hind III fragment of FIV LTR (−106 to −65 bp) including the C/EBP site upstream of the human immunodeficiency virus (HIV) LTR, an approximately fourfold increase in CAT activity was observed, whereas the insertion of mutated versions of the C/EBP site failed to

increase the CAT activity (Fig. 2). These results indicated that the C/EBP site is critical for the basal promoter activity of the FIV LTR in addition to the AP-1 and ATF sites and that the C/EBP site is sufficient to confer the enhancer activity on a promoter lacking the regulatory sites in the FIV LTR.

Effect of the deletion of the C/EBP site in the FIV LTR on viral replication

We examined further the effect of deletion of the C/EBP site on viral replication by constructing mutant IMCs of FIV (pdTM and pd14), which lack the C/EBP sites in the LTRs, and then introducing the mutant viral DNAs into CRFK and MYA-1 cells. Virus production was measured by the RT activity assay. Two days after transfection, the RT activities of the culture supernatants from CRFK cells transfected with the mutated IMCs were only about 1/15 the amount of those from the cells transfected with the wild-type IMCs (Fig. 3A). Similar results were also obtained in MYA-1 cells (Fig. 3B). When equivalent amounts (1.0×10^4 cpm of RT activity) of cell-free progeny viruses produced by the clone (pCtTM or pdTM) in CRFK cells were inoculated onto MYA-1 cells, the deletion of the C/EBP site also caused a marked delay of productive infection in FIV-infected MYA-1 cells (Fig. 3C). The wild-type virus replicated in MYA-1 cells efficiently, whereas the mutant virus lacking the C/EBP site was not detectable until 28 days postinfection (Fig. 3C). The cytopathogenic activity of the mutant virus was also lower than that of the wild-type virus (Fig. 3D). The specificity for the FIV infection was confirmed by the IF assay using an anti-FIV cat serum (data not shown). These data showed that the C/EBP site is important not only for basal promoter activity of the FIV LTR but also for replicative ability of FIV.

Existence of the C/EBP site binding activity in MYA-1 and CRFK cells

To determine whether the C/EBP site of the FIV LTR really functions in factor recognition, the gel-mobility-shift assay was performed using extracts from MYA-1 and CRFK cells. When a ds oligonucleotide containing the C/EBP site was labeled and incubated with extracts from CRFK cells, two binding complexes, CI and CII, were observed (Fig. 4), while only one binding complex, CI, was observed in MYA-1 cells. To assess the specificity of the binding to the C/EBP site, competition experiments were performed using homologous or heterologous unlabeled competitor DNA. The binding was competed by addition of the homologous unlabeled competitor. The competition was not demonstrated with the heterologous oligonucleotide containing the Sp-1 binding site. These results indicated that nuclear factors bind to the C/EBP site and the binding patterns of nuclear extracts from .
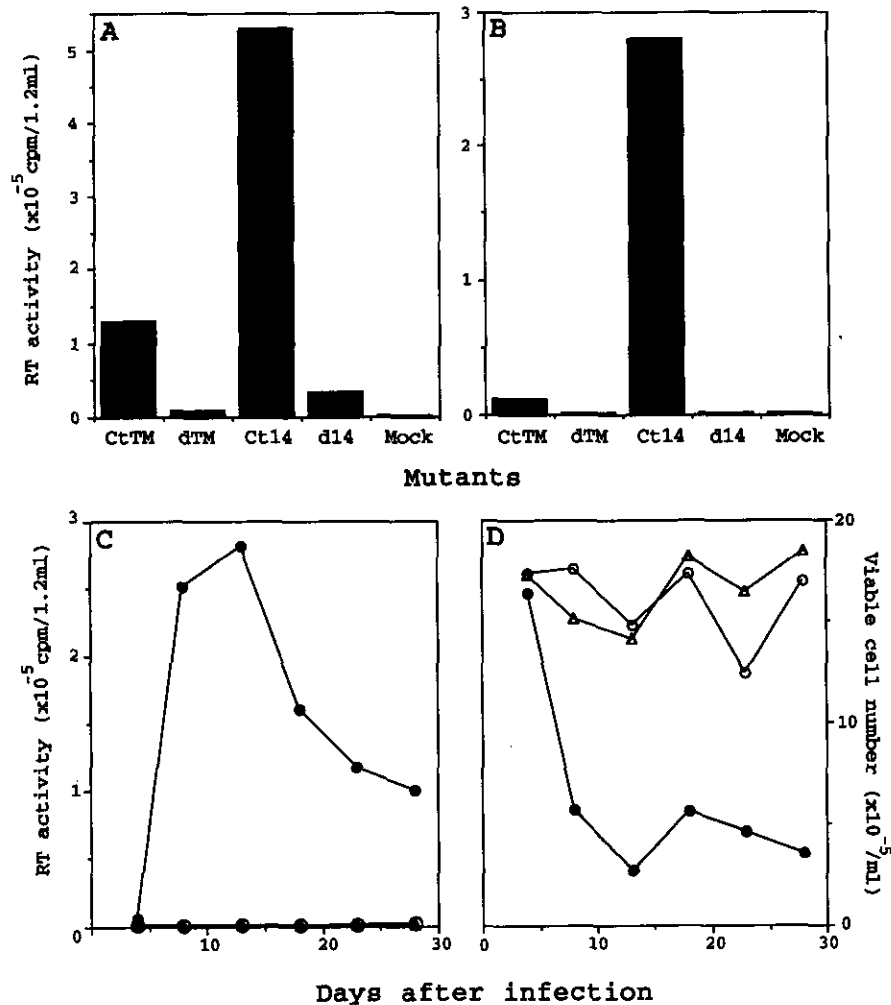
FIG. 3. Effect of deletion of the C/EBP site in the FIV LTR on viral replication. CRFK (A) and MYA-1 (B) cells were transfected with 20 μg of the infectious molecular clones containing intact and deleted LTRs. The culture supernatants from transfected CRFK and MYA-1 cells were harvested 2 and 6 days after transfection, respectively. (C) MYA-1 cells were mock-infected (Δ) and infected with viruses derived from pCtTM (●) and pdTM (O). The culture supernatants from infected MYA-1 cells were harvested at the indicated times. Virus production was monitored by measuring RT activities of the culture supernatants. Cytopathogenic activity was monitored by dye-exclusion test (D).

MYA-1 and CRFK cells to the site are different from each other.

The C/EBP site in the FIV LTR responds to inhibition of FIV LTR-directed gene expression by PRV ICP4

We previously demonstrated that alphaherpesvirus ICP4s significantly inhibit FIV LTR-directed gene expression in CRFK and fcwf-4 cells (Kawaguchi et al., 1994). To identify which functional regions within the FIV LTR respond to the inhibition by alphaherpesvirus ICP4, a cotransfection experiment was performed using the site-specific mutants of the FIV LTR and a PRV ICP4 expression vector. As shown in Table 1, the PRV ICP4-mediated FIV LTR inhibition was observed by deletion of AP-1 or ATF site. In contrast, when the C/EBP site was deleted, the inhibition of the CAT activity was completely abolished.

Effect of the PRV ICP4 on heterologous promoters fused to the FIV C/EBP site

To assess further the ability of the C/EBP site within the FIV LTR to confer the inhibition by the PRV ICP4, we performed cotransfection experiments using the PRV ICP4 expression vector and the reporter plasmid which contained the sequence including the C/EBP site of the FIV LTR upstream of the HIV LTR (Fig. 2). When the FIV C/EBP site was inserted 5' to the HIV LTR, the PRV ICP4 inhibited CAT expression, while the insertion of the mutated FIV C/EBP site failed to inhibit the gene expression (Table 2). These results suggested that the C/EBP site in the FIV LTR is required for response to the PRV ICP4-mediated negative regulation of the FIV LTR.

DISCUSSION

This is the first report describing the effects of specific mutation of the C/EBP site within the FIV LTR on viral

A

C/EBP GCTTAACCGCAAAACCACATCC

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          C/EBP   C/EBP
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              Sp-1
Sp-1  ATTCGATCGGGGCGGGGCGAGC
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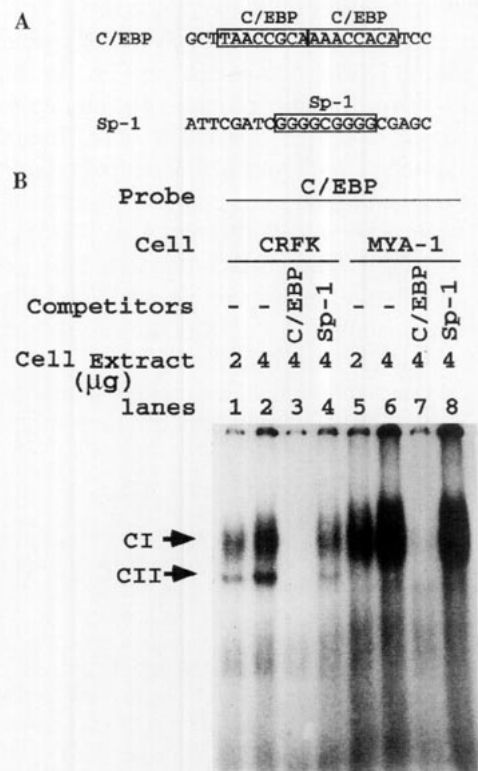
B

FIG. 4. Gel-mobility-shift assays using nuclear extracts from CRFK and MYA-1 cells. (A) The sequences of the oligonucleotides used are shown. The C/EBP and Sp-1 sites are boxed. (B) Specific binding of nuclear factors from CRFK and MYA-1 cells to the C/EBP site of the FIV LTR. End-labeled oligonucleotide was incubated with 2 μg (lanes 1 and 5) or 4 μg (lanes 2 to 4 and 6 to 8) of CRFK and MYA-1 cell extracts. Specificity of the retarded complexes was analyzed by using a set of competitors. The retarded complexes, CI and/or CII, are competed out by the unlabeled competitor (100-fold molar excess in lanes 3 and 7), but the nonspecific competitor is unable to compete (lanes 4 and 8).

replication and LTR-directed gene expression. As shown in Figs. 1 and 3, the effect of site-specific mutation of the C/EBP site reduced the basal promoter activity much more than those of the AP-1 and ATF sites in CRFK cells, and the mutation of the C/EBP site also significantly diminished viral replication in both CRFK and MYA-1 cells. These results suggested that FIV replication and LTR-directed gene expression are strongly dependent on the interaction of the factor(s) that binds to the C/EBP site within the FIV LTR. The demonstration of decreases in both LTR-directed gene expression and viral replication is important because almost all information about the FIV LTR function comes from studies in which DNA constructs containing the LTR linked to reporter genes were tested in some cell lines (Kawaguchi et al., 1992; Sparger et al., 1992; Thompson, F. J., et al., 1994); however, the function of the regulatory elements within the FIV LTR and the interaction of virus-encoded and cellular proteins with the elements during viral replication cannot be evaluated in such systems. Actually, we previously

TABLE 1

Effect of PRV ICP4 on Various Deletion Mutants of the FIV LTR

Plasmids		Expt 1	Expt 2	Expt 3	Average[a] of fold change ± SD
pTM1 CAT	+pRVSV	24964	22833	22321	
(2 μg)	+pRVPRIE	7276	7290	6328	0.30 ± 0.017
pdAP1 CAT	+pRVSV	10456	7954	4610	
(2 μg)	+pRVPRIE	4384	2920	1922	0.40 ± 0.024
pdEBP CAT	+pRVSV	1455	1681	684	
(2 μg)	+pRVPRIE	3470	2484	1587	2.1 ± 0.40
pdATF CAT	+pRVSV	13813	9941	8031	
(2 μg)	+pRVPRIE	3794	3058	2026	0.28 ± 0.025
pHd CAT	+pRVSV	200	112	102	
(2 μg)	+pRVPRIE	151	113	103	0.92 ± 0.11

[a] Determined by comparison of the CAT activity associated with the target plasmid in the presence of the indicated effector plasmid versus that in the presence of pRVSV.

reported that internal deletion of AP-1 and AP-4 sites of the FIV LTR resulted in the reduction of the basal promoter activity despite the fact that virus lacking these sites replicated efficiently in T-lymphoblastoid cell lines and CRFK cells, with kinetics that are similar to those of the wild-type virus (Miyazawa, et al., 1993).

Viral regulatory proteins such as PRV ICP4 and herpes simplex virus type 1 (HSV-1) α-TIF are often used as convenient tools to probe viral and cellular mechanisms of transcriptional regulation (Abmayr et al., 1988; Cleary et al., 1992; Cromlish et al., 1989; Yuan et al., 1989). In the present studies, it should be noted that the C/EBP site within the FIV LTR responds to the inhibition of FIV LTR-directed gene expression by the PRV ICP4 in CRFK cells. These data suggest the possibility that the C/EBP site might have a potential function in the negative regulation of FIV gene expression, in addition to the fact that

TABLE 2

Effect of PRV ICP4 on Insertion of the C/EBP Site of the FIV LTR into the HIV LTR

Plasmids		Expt 1	Expt 2	Expt 3	Average[a] of fold change ± SD
pH1o CAT	+pRVSV	2209	1568	2666	
(0.5 μg)	+pRVPRIE	4425	3610	3723	1.9 ± 0.37
pH1EBP CAT	+pRVSV	10304	8100	11631	
(0.5 μg)	+pRVPRIE	3939	3556	3109	0.37 ± 0.071
pH1dEBP CAT	+pRVSV	1881	1606	1826	
(0.5 μg)	+pRVPRIE	2716	3022	3889	1.8 ± 0.29

[a] Determined by comparison of the CAT activity associated with the target plasmid in the presence of the indicated effector plasmid versus that in the presence of pRVSV.

the site is critical for the positive regulation of FIV gene expression and replication. Interestingly, Thompson, J. R., et al. (1994) also suggested that the C/EBP site in the p41 promoter of human herpesvirus 6 (HHV6) played roles in both activation of the p41 basal promoter activity in HHV6-infected cells and repression of the activity in uninfected cells. Although there is no direct evidence for the binding of C/EBP family members to the C/EBP site within the FIV LTR, it is likely that these bind to this site, because the site matches closely the binding site for the SV40 core enhancer protein, C/EBP (Miyazawa et al., 1994), and the binding was strongly competed by an oligonucleotide based on the SV40 sequence as reported by Thompson, F. J., et al. (1994). Among the C/EBP family members, there are both transcriptional activators and repressors. The repressor LIP (Descombes and Schibler, 1991) is a competitive inhibitor, and the CHO-10 (Ron et al., 1992) is a dominant-negative inhibitor of the transcriptional activators of the C/EBP family members such as C/EBP (Johnson et al., 1987) and NFIL6 (Akira et al., 1990). The ultimate on-off decision of gene expression may be dependent on an averaged effect of the activators and repressors existing at various concentrations as reported by Descombes and Schibler (1991). It might be possible that the change of the activators/repressors ratio mediated by PRV ICP4 expression occurred and resulted in the inhibition of FIV LTR-directed gene expression.

Gel-shift analysis (Fig. 4) shows a difference in the binding patterns of nuclear extracts from CRFK and MYA-1 cells to the C/EBP site in the FIV LTR. The oligonucleotide containing the C/EBP site formed the specific complex, CI, with the nuclear extract present in MYA-1 cells, whereas the same (CI) and the additional (CII) complexes were detected with the nuclear extract from CRFK cells. These results suggested that the C/EBP site within the FIV LTR is recognized by more than one nuclear factor. Similarly, the homologous C/EBP site in a murine retrovirus LTR also is known to be recognized by multiple cellular proteins and to be important for T-lymphocyte specificity (Boral et al., 1989; Thornell et al., 1988). It is unknown whether the difference shown in Fig. 4 reflects on FIV gene expression at present. Further analysis will be interesting and needed to clarify this subject. The information will also provide insight into the transcriptional selectivity of the C/EBP site.

The ICP4 of alphaherpesvirus has been found to be one of the most important regulators in control of alphaherpesvirus gene expression (Hayward, 1993). The ICP4 is also characterized as a multifunctional regulator that transactivates a variety of viral and cellular promoters and down-regulates its own and latent-associated promoters (Hayward, 1993; Smiley et al., 1991). To our knowledge, only the promoters with ICP4 binding sites near their mRNA start sites are down-regulated by ICP4 (Hayward, 1993). It is, therefore, quite unique that FIV LTR-directed gene expression is inhibited by ICP4s. Our observation that the repression of FIV LTR mediated by ICP4 is via the C/EBP site within the FIV LTR suggests that the ICP4 may directly or indirectly interact with the factor(s) which binds to the C/EBP site. The ICP4 is thought to interact with cellular transcription factors, because the activation of some promoters by ICP4 varied with cell types (Everett, 1988). Smith et al. (1993) reported that ICP4 of HSV-1 interacts with transcriptional preinitiation complex, TATA-binding protein, and TFIIB. Thus, our data provide insight into not only the regulation of FIV gene expression but also functions of ICP4. The understanding of these mechanisms may help to clarify pathogenesis of both lentivirus and alphaherpesvirus.

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