Cis- and trans-regulation of feline immunodeficiency virus: identification of functional binding sites in the long terminal repeat

Fiona J. Thompson, John Elder and James C. Neil

MRC Retrovirus Research Laboratory, Department of Veterinary Pathology, University of Glasgow, Bearsden, Glasgow G61 1QH, U.K. and Scrips Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037, U.S.A.

Nuclear protein binding sites in the long terminal repeat (LTR) of feline immunodeficiency virus (FIV) were identified by the method of DNase I footprinting. Using nuclear protein extracts from a feline T lymphoma cell line, several discrete footprints were generated upstream of the transcriptional initiation site (-50 to -150). The specificity of protein binding was examined by competition with oligonucleotides representing consensus DNA binding sites for known transcription factors. Binding to AP-1 (-124) and ATF (-58) motifs was observed, with cross-competition between these sites. A strong footprint signal was also detected over a tandemly repeated C/EBP motif (-94, -86) and an adjacent weaker footprint was found to be specific for an NF1 motif (-72/-63). The effect on FIV LTR promoter activity of progressively deleting these nuclear factor binding sites was examined by linking LTR deletion mutants to the chloramphenicol acetyltransferase (CAT) gene. Deletion of the AP-1 site caused a 10- to 25-fold loss of CAT activity whereas deletion past the ATF site reduced activity virtually to background levels. The effects of deleting the C/EBP and NF1 sites were less marked and varied according to cell type. Trans-activation of the LTR was assayed using constructs linked to a CAT reporter gene. The full-length FIV LTR was not significantly trans-activated. However, the expression of a deleted LTR construct lacking the AP-1/AP-1 site but retaining C/EBP and ATF sites was partially restored by co-infection with FIV or by cotransfection with an infectious molecular clone of FIV (FIV-PPR). These results show that host transcription factors responsive to cellular activation have a major role in regulating FIV expression, and suggest that virus-coded trans-activators acting through U3 may play a role in some cellular environments.

Introduction

Feline immunodeficiency virus (FIV) was first identified only 7 years ago (Pedersen et al., 1987) but retrospective serological surveys have revealed this agent to be a long-established and ubiquitous pathogen of free-range cat populations (Hosie et al., 1989). Although FIV is not closely related to any previously identified virus, nucleotide sequence homology and genetic organization favour its classification within a subgroup of animal lentiviruses including equine infectious anaemia virus (EIAV) and visna virus (Talbott et al., 1989; Olmsted et al., 1989a, b; Phillips et al., 1990). In contrast to other members of this subgroup, FIV replicates readily in T lymphocytes and induces pathological changes closely similar to those seen in human AIDS. The important determinants of FIV host range and pathogenesis have yet to be identified, although it appears that FIV recognizes a host cell surface receptor distinct from CD4 (Hosie et al., 1993).

The lentiviruses are characterized by a complex life cycle which entails the rapid activation of transcriptionally inert proviruses to high levels of expression (Cullen, 1991). This process is mediated by a complex regulatory cascade involving trans-acting cellular and viral factors and their cis-acting viral target sequences. The primary targets for transcriptional control are the proviral long terminal repeats (LTRs), which also carry signals directing the correct processing of viral RNA transcripts, the faithful reverse transcription of the genome and the integration of proviral DNA into the host cell genome (Temin, 1981). Despite their common functional roles, LTR elements from viruses that are not members of a close family generally display little sequence relatedness. This apparent lack of conservation may be a consequence of the structure of the regulatory elements which are modular arrays of binding sites for factors that bind specifically to short DNA target sequences (Speck & Baltimore, 1987; Ondek et al., 1988). Identification and functional characterization of these
binding sites are therefore essential steps towards the understanding of the transcriptional controls operating on the virus and the establishment of functional relationships between lentiviruses.

Previous reports have highlighted a number of motifs in the FIV LTR that are potential sites for protein binding (Olmsted et al., 1989b; Talbott et al., 1989; Phillips et al., 1990; Miyazawa et al., 1991) and mutational analysis has shown that putative AP-1 and ATF sites are required for full basal promoter function (Sparger et al., 1992). However, there has until now been no direct analysis of nuclear proteins interacting with the FIV LTR. We undertook this analysis with DNase I footprint and deletion analysis of the LTR of a typical FIV field isolate, FIV-UK8. Our analysis reveals a distinctive and complex array of binding sites for at least three distinct families of transcription factors.

In accord with previous studies (Sparger et al., 1992; Kawaguchi et al., 1992), we found high basal expression but no significant trans-activation of reporter genes linked to the full-length FIV LTR. However, truncated promoter constructs with low basal expression showed significant restoration of activity by FIV in trans, suggesting that trans-activation of the FIV promoter may be operative in some cellular environments.

Methods

Cell lines and viruses. CrFK cells were originally derived from the renal cortical cells of a normal domestic kitten (Fischinger et al., 1973). The ID10 subclone of CrFK, which is highly permissive for the Petaluma strain of FIV (Olmsted et al., 1989a), was kindly provided by P. Andersen (Idexx Corporation, Portland, Maine, U.S.A.). F422 cells are lymphoid tumour cells which were established from a thymic lymphosarcoma of a kitten inoculated with feline leukaemia virus (Rickard et al., 1969). AH927 is an immortal feline cell line of fibroblast origin (Rasheed & Gardner, 1980). Nuclear extracts were generated from these cells as described (Fulton et al., 1990; Plumb & Goodwin, 1988). For transfection experiments, CrFK and AH927 cells were used as well as the human carcinoma cell line HeLa (Gey et al., 1952). FIV strains used in this study were a series of isolates from the U.K. as previously described (Ribgy et al., 1993) and the molecular clones of FIV-Petaluma (34TF10) (Talbott et al., 1989) and FIV-PPR (Phillips et al., 1990).

Oligonucleotides. Oligonucleotides were produced in-house on an Applied Biosystems 318A DNA synthesizer or purchased from commercial sources as specified in the figure legends.

LTR sequences. The U.K. isolates of FIV used in this study have been described previously (Ribgy et al., 1993), were obtained from a nationwide survey of FIV seroprevalence (Hosie & Jarrett, 1990) and propagated in F422 feline T lymphoma cells. The LTR sequence was amplified by PCR using primers based on the FIV-Petaluma sequence (Olmsted et al., 1989b; Talbott et al., 1989), as shown underlined in Fig. 1. The gel-purified PCR products were ligated to HincII-digested pIC-19R (Marsh et al., 1984) and the DNA sequence was determined on both strands using the dideoxynucleotide chain-termination technique (Sanger et al., 1977). Reaction mixtures contained 1 μg of Hirt supernatant DNA, 1 μg of each primer (1 mg/ml), 10 μl of 10× reaction buffer, 500 nm deoxynucleotides, 0.5 units of recombinant Taq polymerase and water to a volume of 100 μl. Amplification was achieved with 30 cycles of denaturation for 1 min at 91 °C, annealing at 45 °C for 1 min and polymerization at 72 °C for 2 min. Primers used for amplification were based on the FIV-Petaluma sequence (Olmsted et al., 1989b): 5'-TGGGATGAGTATGGAACCTGAAGA 3'; 3'-SmaI-BglII-BamHI

PCR mutagenesis. Deletion mutants of the FIV-UK8 LTR were generated by PCR amplification with a fixed 3'-terminal primer (5'-TGGCAGGTTCGCGCCGATTCTC 3') and a series of internal 5' primers as follows: -176: 5'-GCTTATGAAGTAAGACTGTC 3'; -147: 5'-ACGATTAGGAAAACAGCTGA 3'; -126: 5'-GCACTGACTGATACAGCTG 3'; -113: 5'-TAAAGGCTAGCAGCTG 3'; -102: 5'-AAGCTTGCCAAATGAG 3'; -74: 5'-TTTGCTCCACTGTA 3'. The amplified fragments were cloned into the Smal site of plasmid pIC-19R and then cloned as BglII–BamHI fragments into the BamHI site of the pCAT12 plasmid which contains a functional Tet chloramphenicol acetyltransferase (CAT) gene but lacks a eukaryotic promoter (Spandidos & Riggio, 1986). Correct orientation was verified by sequence analysis of the subcloned fragments.

DNase I footprinting. Crude nuclear extracts were prepared essentially as described previously (Fulton et al., 1990; Plumb et al., 1991). The construct pIC-FIVLTR-UK8 was digested with BamHI, treated with calf intestinal phosphatase, and 5' end-labelled with [γ-32P]ATP and T4 polynucleotide kinase. The insert was then isolated after secondary digestion with BglII. Footprint protection assays were performed as outlined previously (Plumb & Goodwin, 1988). In short, assay mixtures contained 0 to 80 μl of nuclear extract, 1 to 5 ng of end-labelled LTR fragment and 1 μg of poly(dI-dC), with or without 100 ng of double-stranded competitor oligonucleotide DNA in a final volume of 100 μl. After limited DNase I digestion, nucleic acids were purified and resolved by denaturing polyacrylamide gel electrophoresis and autoradiography. Markers were prepared from the same fragment by the chemical sequencing method of Maxam & Gilbert (1980), using the G- and G+A-specific reactions.

Transfection and CAT assays. FIV LTR-CAT constructs were transfected into a range of cell lines including FIV-susceptible CrFK cells and non-susceptible feline fibroblast (AH927) or HeLa cells using the calcium phosphate precipitation method (Graham & van der Eb, 1973). Cell extraction and assay of CAT activity were performed according to established procedures (Gorman et al., 1982). Unmodified substrate and acetylated products were detected by thin-layer chromatography and quantified by liquid scintillation counting.

Results

Protein binding sites in the FIV LTR U3 domain: DNase I footprint analysis reveals three major binding domains

Fig. 1 shows an alignment of the LTR sequences from eight isolates of FIV including five newly derived sequences from U.K. isolates. A number of motifs corresponding to transcriptional control signals and potential binding sites are underlined, as are the primers used to amplify the U.K. viral sequences. We wished to determine whether the binding motifs represented bona fide binding sites and to examine their significance for
the transcriptional regulation of FIV gene expression. Nuclear protein extracts were prepared from various feline cell lines and these were used for in vitro footprinting protection assays with end-labelled FIV LTR fragments. Fig. 2 shows footprint protection of the FIV-UK8 LTR using nuclear extracts from F422 feline T lymphoma cells which support the replication of a broad range of FIV strains. Three broadly defined binding sites were identified in the U3 domain, and the location of these is indicated by the dashed line under the sequence in Fig. 1.

To investigate further the specificity of binding to these sites in the FIV LTR, we carried out binding experiments in the presence of competitor oligonucleotides based on consensus binding sequences which appeared to be included within the footprint (Fig. 2).
Site 1: AP-4/AP-1
The broad footprint covering −135/−115 contains adjacent motifs corresponding to binding sites for transcription factors AP-4 and AP-1. Binding to the FIV site was efficiently competed by oligonucleotides based on the consensus AP-1 site and an example is shown in Fig. 2 (lane 2). Partial inhibition was also observed with an oligonucleotide based on an ATF site from the adenovirus E3 promoter (lane 6) and a similar motif within the FIV LTR (lane 7). This cross-competition presumably reflects the similarity between the core recognition sequences (TGACGT and TGACTCA) and their interaction with an overlapping set of heterodimeric protein complexes (Hai et al., 1988). As shown in Table 1, site 1 in the FIV LTR is similar to the adjacent AP-4 and AP-1 sites which act cooperatively to regulate the simian virus 40 (SV40) late promoter (Mermod et al., 1988). There is also a striking similarity between the FIV binding site and a central element of the visna virus LTR promoter which is essential for basal promoter activity and for trans-activation (Hess et al., 1989).

Although the site 1 footprint extends into the AP-4 motif, we found that a competitor oligonucleotide based on the AP-4 site had no discernible effect on the footprint (lane 1). It is possible that the footprint at this site is due exclusively to AP-1 binding; AP-4 binding activity may be insufficiently abundant or stable to extraction to register in the footprint assay. A cooperative binding reaction would seem less likely since no such interaction was observed with purified factors acting on the SV40 A domain (Mermod et al., 1988). Resolution of this issue will require further characterization of the protein complexes interacting with site 1 by electrophoretic mobility shift analysis.

Comparison of the LTR sequences of five U.K. isolates with published U.S. PPR and Petaluma and Japanese (TM1) FIV isolates (Fig. 1) showed almost complete conservation of both AP-1 and AP-4 motifs. The only departure from consensus binding sites is seen in the AP-4 site of FIV-Petaluma while a single base difference in the TM1 AP-1 site does not disturb the consensus (Lee et al., 1987).

Site 2: C/EBP
A strong footprint was found spanning tandemly repeated motifs (−80/−90) which match closely to the binding site for the SV40 core enhancer binding protein C/EBP (Table 1) (Landschultz et al., 1988), and binding was strongly competed by an oligonucleotide based on the SV40 sequence (Fig. 2, lane 3). The sequence here is similar to a site in the EIAV LTR which is tandemly repeated in some strains (Derse et al., 1987). The homologous C/EBP site in mammalian type C retroviral LTR enhancers is known to be bound by multiple
Table 1. Consensus binding sites in the FIV LTR: alignment with related sites in other viruses

<table>
<thead>
<tr>
<th>Site</th>
<th>Source</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-4/AP-1</td>
<td>Consens</td>
<td>TCAGCTGTTG...  TGACTCA</td>
<td>Mermod et al. (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C  C  T  G</td>
<td></td>
</tr>
<tr>
<td>SV40</td>
<td></td>
<td>CCAAGCTGGAGATGCTGCTCA</td>
<td>Mermod et al. (1988)</td>
</tr>
<tr>
<td>FIV-UK8 (-135, -118)</td>
<td></td>
<td>ACAGCTGATCCA TGACTCA</td>
<td></td>
</tr>
<tr>
<td>Visna virus (-65, -47)</td>
<td></td>
<td>GCAGCTGAAGGT TGACTCA</td>
<td>Gabuzda et al. (1989)</td>
</tr>
<tr>
<td>CAEV* (-73, -56)</td>
<td></td>
<td>TCAGCTGAAGGT TGACTCA</td>
<td>Hess et al. (1989)</td>
</tr>
<tr>
<td>C/EBP</td>
<td>Consens</td>
<td>TGTTGGTATT</td>
<td>Landschultz et al. (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGTTGAAG</td>
<td></td>
</tr>
<tr>
<td>FIV-UK8 (1)</td>
<td></td>
<td>TGGGTTTATT</td>
<td>Derse et al. (1987)</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>TGGGTTTATT</td>
<td></td>
</tr>
<tr>
<td>EIAV</td>
<td></td>
<td>TGGGTTTATT</td>
<td></td>
</tr>
<tr>
<td>Mo-MuLV*</td>
<td></td>
<td>TGGGTTTATT</td>
<td>Boral et al. (1989)</td>
</tr>
<tr>
<td>FeLV*</td>
<td></td>
<td>TGGGTTTATT</td>
<td>Fulton et al. (1990)</td>
</tr>
<tr>
<td>NF1</td>
<td>Consens</td>
<td>TGG (NG_2) GCCA</td>
<td>Gronostajski (1986)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGG (NG_2) GCCA</td>
<td></td>
</tr>
<tr>
<td>FIV-UK8</td>
<td></td>
<td>TGG (NG_2) GCCA</td>
<td></td>
</tr>
<tr>
<td>FeLV</td>
<td></td>
<td>TGG(NG_2) GCCA</td>
<td>Fulton et al. (1990)</td>
</tr>
<tr>
<td>Mo-MuLV</td>
<td></td>
<td>TGG(NG_2) GCCA</td>
<td>Thornell et al. (1988)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td></td>
<td>TGG(NG_2) GCCA</td>
<td>Gronostajski (1986)</td>
</tr>
<tr>
<td>ATF</td>
<td>Consens</td>
<td>GTGACGT</td>
<td>Lin &amp; Green (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A  A</td>
<td></td>
</tr>
<tr>
<td>FIV-UK8</td>
<td></td>
<td>GTGACGT</td>
<td>Katoh et al. (1989)</td>
</tr>
<tr>
<td>BLV*</td>
<td></td>
<td>GTGACGT</td>
<td></td>
</tr>
<tr>
<td>Adenovirus E4</td>
<td></td>
<td>GTGACGT</td>
<td>Lin &amp; Green (1988)</td>
</tr>
</tbody>
</table>

* CAEV, Captive arthritis-encephalitis virus; Mo-MuLV, Moloney murine leukaemia virus; FeLV, feline leukaemia virus; BLV, bovine leukaemia virus. The other abbreviations are given in the text.

cellular proteins including the T lymphoma-specific SEF-1/SBP factor (Thornell et al., 1988; Boral et al., 1989). It is likely that the FIV C/EBP-like site is recognized by more than one cell-type nuclear factor and in accord with this suggestion we noted subtle differences in the footprints generated by the T lymphoma and fibroblast cell extracts (not shown). The tandem C/EBP recognition sequences represent an interrupted stretch of completely conserved sequence in all strains of FIV that have been sequenced (Fig. 1).

Site 2b/3a: NF1

A third, weaker footprint was resolved by competition with an oligonucleotide based on the adenovirus NF1 site and showed that an additional footprint was present between the stronger protection at sites 2 and 3 defined in our initial analysis. Although site 3a also contains a CCAAT sequence, oligonucleotides containing only this ‘half NF1 site’ did not compete efficiently in the footprint assay and it would appear that the abundant CCAAT binding proteins (Santoro et al., 1988) do not recognize this site. The conservation of this motif among FIV strains is less clear, however, since changes at bases known to be crucial for NF1 binding (Gronostajski, 1986) were seen in the FIV-UK2 and FIV-Petaluma strains. The effect of these natural sequence variations on protein binding and promoter function have yet to be examined.

Site 3: ATF

The site 3 footprint at −50/−60 centres on a consensus ATF binding site. Binding could be competed out with oligonucleotides based on the ATF site of the adenovirus E4 promoter (Fig. 2, lane 6). Strong competition was also noted with an oligonucleotide homologous to the AP-1 site (lane 2), showing reciprocal competition between these sites. An oligonucleotide based on the FIV site 3 was also able to compete for binding at site 1 as well as its homologous site (lane 7). A five base central core of the ATF motif (TGACG) is completely conserved among FIV isolates, with variation only in flanking bases in some U.K. field isolates (Fig. 1).

Other features of the FIV LTR sequence

Other recognized elements in the FIV LTR sequence that were found to be completely conserved between isolates were the TATA box at −28 and the AATAAA polyadenylation signal at +53 (Fig. 1). A second TATA motif at −54 which was previously noted in the FIV-Petaluma isolate was not conserved in the TM-1, PPR, UK5 and UK14 sequences. A putative NF-κB motif which was noted in the FIV-Petaluma LTR (Olmsted et al., 1989b; Talbott et al., 1989) was also variable between strains and we did not detect protein binding to the homologous site in the FIV-UK8 sequence (not shown).
Fig. 3. Deletion mutants of the FIV LTR promoter. The figure shows the FIV LTR with the locations of sequence motifs and the three major binding sites identified (Fig. 1) and DNase I footprint protection (Fig. 2). The panel of deletion mutants was generated by PCR mutagenesis as described in the text.

In contrast to feline leukaemia virus (Fulton et al., 1990) and visna virus (Hess et al., 1989), in FIV we found no strain-specific duplications of the enhancer domain. The most divergent LTR sequence is the TM-1 isolate which is only 84% identical to that of FIV-UK8 and has a seven base pair insertion at +61 including a perfect repeat of the adjacent six bases (Fig. 1). The U.K. sequences show 94 to 99% sequence match over the entire LTR; the U.S. isolates were only slightly less closely related (93 to 95%). These results are in accord with sequence comparisons of the same isolates based on the FIV env and gag genes which suggested that the Japanese isolate belongs in a separate FIV subgroup (Rigby et al., 1993).

Deletion analysis of the FIV LTR: AP-1 and ATF motifs are essential for basal LTR promoter function

As a further test of the functional importance of the binding sites we identified in the footprint assay, a panel of mutants was constructed with progressive deletion from the 5' end of the LTR. This was achieved by specific amplification with PCR primers corresponding to the 3' end of the LTR and a series of intermediate positions intersecting the identified binding sites and other putative regulatory elements (Fig. 3). The amplified fragments were cloned into plasmid pCAT12 (Spandidos & Riggio, 1986) upstream of the CAT reporter gene and those with the desired orientation were selected. The wild-type and deletion mutant LTR–CAT plasmids were transfected into a range of cell lines using the calcium phosphate precipitation procedure (Graham & van der Eb, 1973). After 48 h, cytoplasmic extracts were prepared and CAT activity was measured. Transfection efficiencies were checked by re-extracting DNA from the transfected cells and measurement by Southern blot analysis. Also, in some cases co-transfection was performed with a plasmid

Fig. 4. Relative activity of CAT reporter genes linked to FIV LTR deletion mutants in CrFK, AH927 and HeLa cells (a to c respectively). Assays were performed as described in Methods, in the linear range of CAT activity relative to input DNA. Results were an average of at least three determinations and are expressed relative to the full-length (wild-type, wt) FIV LTR. Standard deviations are represented by extension bars.
carrying a functional β-galactosidase gene which served as an internal standard.

Fig. 4 is a graphic representation of the relative CAT activities of the mutant panel in various cell lines, normalized to the full-length LTR control (1:00). The cell lines compared here were feline fibroblasts either permissive (CrFK) or resistant (AH927) to FIV infection and also heterologous cells refractory to FIV infection (HeLa). Since none of the FIV-susceptible feline T cell lines yielded measurable CAT activity by any of the available transfection procedures these could not be used for our transcriptional analysis.

The deletion mutants showed some consistent properties regardless of cell type. The effect of deleting the AP-4/AP-1 motif was very marked, particularly when the core of the binding site was removed (−113) (Fig. 3 and 4). This deletion resulted in a 10- to 25-fold loss of activity relative to the wild-type LTR (Fig. 4). Also notable was the virtual disappearance of promoter activity when site 3 (ATF site) was deleted (−47). An intermediate deletion of site 2 [(−68); both C/EBP motifs and the 5' half of the NF1 site] had less dramatic effects which also varied according to cell type. In CrFK cells the effect of the deletion was to reduce activity further whereas in AH927 and HeLa cells a slight increase in activity was recorded. A further cell-type difference was seen in a cis-acting negative regulatory element between −176 and −147 which appeared to be operative in HeLa cells but was much less marked in the feline fibroblast cells. The −147 deletion mutant was almost threefold more active than the full-length LTR in HeLa cells, but showed only slightly increased activity in CrFK and AH927 cells (Table 2). However, the overall levels of activity were low in HeLa cells and the significance of this effect is as yet unclear.

Trans-activation of the FIV-UK8 LTR in CrFK cells

Previous studies have shown that the full-length LTR of FIV is only very weakly trans-activated, if at all, by co-transfection with FIV clones or subgenomic components (Sparger et al., 1992). However, the possibility remained that trans-activation might be masked by the high basal activity of the FIV LTR in established cell lines. The full-length FIV LTR–CAT construct and the deletion mutants were tested for trans-activation in two ways. First, CrFK cells infected with FIV-UK8 were trans-
fected with the LTR–CAT plasmid panel and activity was compared to that recorded in uninfected cells. In a second approach the CAT reporter constructs were co-transfected with full-length infectious molecular clones of FIV, FIV-Petaluma (34TF10) and FIV-PPR (Phillips et al., 1990; Talbott et al., 1989).

We found no significant increase in the activity of the full-length FIV-UK8 LTR in infected cells (Fig. 5c). However, a consistent observation was the restoration of activity of the −113 deletion mutant almost to wild-type levels in cells infected with FIV-UK8 (Fig. 5c) or co-transfected with FIV-PPR (Fig. 5b). This effect was lost on further removal of the tandem C/EBP sites in the −68 deletion mutant.

The relative activities of the LTR deletion mutants co-transfected with FIV-PPR (Fig 5b) gave a remarkably similar pattern to infection with FIV-UK8, but co-transfection with 34TF10 (Fig. 5d) did not significantly alter the pattern for the LTR–CAT transfection alone, suggesting some defect in the 34TF10 clone.

Discussion
We have identified binding sites for multiple transcription factors in the U3 region of the FIV LTR. Deletion mutagenesis showed that these sites play a vital role in basal promoter activity in a variety of cell lines. In particular, AP-4/AP-1 and ATF sites at −120 and −55 respectively are necessary for full basal activity. The full-length FIV LTR was not detectably trans-activated but the activity of a deleted promoter construct lacking the AP-4/AP-1 motif could be partially restored by transactivation.

Our results shed further light on mutational analyses of the FIV LTR performed prior to direct identification of the protein binding sites. Deletion mutants of the FIV-TM1 LTR which were assayed in fcwf-4 feline macrophage-like cells showed a similar 10-fold loss of activity on deletion of the AP-4/AP-1 motif (Kawaguchi et al., 1992). However, in that study deletion of the C/EBP motifs led to reduction of promoter activity to background levels whereas similar deletion mutants in our study retained measurable activity. This apparent inconsistency may be due to the different target cells used in the respective transfection studies or to functional differences between the FIV LTR elements studied. For example, the TM-1 isolate differs from FIV-UK8 in the NF1 motif which was identified as a protein binding site in our footprinting analysis. NF1 is a positive regulator of the feline leukaemia virus LTR (Plumb et al., 1991) and it is conceivable that it plays a similar role in FIV-UK8 but not FIV-TM1.

In another previous study on the FIV-Petaluma (34TF10) strain, mutations introduced into either of the AP-1 or ATF motifs led to eight- to 10-fold decreases in basal promoter activity in CrFK cells. The mutations appeared to have some selective phenotypic consequences since the AP-1 mutation had a greater effect on protein kinase C-mediated responses but the ATF mutation was more deleterious to cAMP responses mediated by protein kinase A (Sparger et al., 1992). In view of the cross-competition between AP-1 and ATF motifs observed here, and the recognition of AP-1 and ATF by an overlapping set of factors (Hai et al., 1988), it might be considered that one intact site could mediate both responses, albeit less efficiently. It would therefore be interesting to examine the effect of mutating both sites that might be expected to have much more drastic consequences for promoter function.

In our studies a weak negative regulatory element was mapped to the 5′ end of U3 in the FIV-UK8 LTR. The effect was slight in AH927 and CrFK cells but much more marked in HeLa cells. FIV 5′ deletion mutants of Japanese and U.S. isolates showed a similar slight increase in fcwf-4 (Kawaguchi et al., 1992) and CrFK cells but a much larger effect in G355-5 brain-derived cells (Sparger et al., 1992). It is interesting to note that the relevant area of the LTR includes a purine-rich sequence (−135/−155) and that negative regulatory activity has been mapped to similar purine-rich binding sites in the human immunodeficiency virus and interleukin 2 gene promoters (Rosen et al., 1985; Siekevitz et al., 1987; Shaw et al., 1988). Cellular factors such as NFAT (Siekevitz et al., 1987) and interleukin binding factor (Shaw et al., 1988) have been shown to interact with such motifs and it has been postulated that transcriptional repression is relieved by degradation of inhibitory proteins or their displacement by positive regulators (Shaw et al., 1988). It would therefore be interesting to examine the effect of deleting this domain on virus replication in vitro and in vivo.

The organization of the FIV LTR is unique but shares common features with the LTR elements of other animal lentiviruses, notably visna virus and EIAV. Binding site 1 in the FIV LTR (−130/−120) centres on a consensus AP-4/AP-1 motif which is closely homologous to regulatory sites in the SV40 late promoter (Mermod et al., 1988) and the visna virus LTR. In visna virus, this site is known to be important for basal activity and serum induction (Hess et al., 1989; Gabuzda et al., 1989) and has been shown to bind the heterodimeric complex of fos and jun proteins (Shih et al., 1992). In the FIV LTR, the AP-4/AP-1 site also appears to be necessary for trans-activation by feline herpesvirus type 1 (Kawaguchi et al., 1992). This suggests a related mechanism of regulation of FIV and visna virus by inducible and regulatable cellular transcription factors. The most striking similarity in organization to the FIV LTR is seen in the WYO12 strain of EIAV which has two C/EBP motifs and an ATF motif in similar spatial relationship.
to the functional TATA box (Derse et al., 1993). EIAV isolates are hypervariable in this domain of the LTR, possibly conferring differences in cell tropism or other biological properties (Carpenter et al., 1993).

The virus-coded trans-activators of HIV and the primate lentiviruses are essential for virus replication but despite the presence in FIV of small open reading frames in similar genomic locations, it has hitherto been unclear whether FIV encodes a TAT-like function. Our results show that significant trans-activation can be detected if the LTR is truncated past the AP-4/AP-1 motif. To explain these observations, we suggest a model in which complete activation of the full-length LTR is achieved via protein–protein interactions that occlude a viral trans-activator. Only when one of the target binding sites is removed by DNA deletion, or one of the necessary factors (e.g. AP-1) is absent, would trans-activation be seen. It should be noted that established cell lines often have aberrant, constitutive expression of AP-1 as a function of their transformed state (Piette et al., 1988) and that such cells may therefore be unrepresentative of FIV-infected cells in vivo. FIV target cells in vivo may conceivably include AP-1-deficient environments in which viral trans-activators could play a significant role.

Further work will be required to identify the FIV product(s) responsible for activating the minimal promoter element. The orf-2 reading frame is a candidate since it is fully open in the PPR and FIV UK8 isolates but prematurely terminates in the 34TF10 clone (Phillips et al., 1990; F. Thompson, unpublished results). However, we have so far been unable to show any activity with constructs containing the isolated orf-2. It is possible that orf-2 does not encode the complete gene product or that a second virus-coded protein is required to form a functional trans-activating complex. Alternatively, there may be other functional differences between the three FIV strains that are responsible for our observations.

There are interesting parallels between FIV and visna virus which, unlike EIAV, lack predicted gene products and RNA structures resembling HIV TAT and TAR (Dorn et al., 1990). Also, the AP-4/AP-1 site of visna virusLTR which is important for basal expression and its TAT trans-activation target is closely homologous to the FIV LTR site which we have characterized. It therefore seemed possible to us that FIV and visna virus share a common trans-activation mechanism involving direct interaction with cellular transcription factors. Although our results support a common role for the AP-4/AP-1 site in regulation of basal expression in response to cellular signals, experiments with truncated LTR promoters do not support a role for this site in viral trans-activation. In contrast, the responsive deleted promoters retain C/EBP and ATF binding sites. Further deletion mutants show that the C/EBP sites are necessary for this response but do not rule out the possibility that the ATF motif is also required. In support of a possible role for this site in trans-regulation, ATF has been shown to bind regulatory proteins involved in cAMP- and E1A-inducible transcription (Mermod et al., 1988; Lin & Green, 1988) and ATF sites are important for regulation by p38tax in the bovine leukaemia virus LTR (Katoh et al., 1989).

In conclusion, this study shows that host transcription factors which are expressed in response to cellular activation bind to the FIV LTR and play a prominent role in regulating its activity. Moreover, this study provides the first direct information on the protein binding sites that control FIV expression and shows the first evidence for a cryptic FIV trans-activator. Further work with viruses mutated in specific factor-binding sites and small open reading frames will help to elucidate the complexities of FIV regulation in vivo, and may assist in the design of attenuated viruses for vaccine application.

The MRC AIDS Directed Programme provided most of the support for this work, including a studentship to F. Thompson. The remainder of the work was supported by grants RO1 AI25825 and RO1 AI28580 (J. E.) from the National Institutes of Health.

References


(Received 24 September 1993; Accepted 19 November 1993)