Feline immunodeficiency virus gene expression: analysis of the RNA splicing pattern and the monocistronic rev mRNA

Keizo Tomonaga,1 Yeon-Sil Shin,1 Masashi Fukasawa,2 Takayuki Miyazawa,1 Akio Adachi3 and Takeshi Mikami1*

1Department of Veterinary Microbiology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, 2Department of Preventive Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852 and 3Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyoku, Kyoto 606, Japan

The transcription pattern of the feline immunodeficiency virus (FIV) genome in a feline CD4+ cell line was examined. In addition to the genomic RNA (9.2 kb), at least five FIV-specific transcripts [5.2, 4.4 (doublet), 1.7 and 1.4 kb] were detected by using subgenomic restriction enzyme fragments of an FIV molecular clone or FIV-specific oligonucleotides as probes. Among these transcripts, the 9.2, 5.2 and 4.4 (doublet) kb mRNAs were not expressed in the cytoplasm of cells transfected with a rev- mutant. To determine the location of splice junctions in the FIV genome, we used PCR to amplify and clone cDNAs corresponding to the viral mRNAs from infected cells. The region between pol and env was found to contain at least two splice donor and three splice acceptor sites. Two splice acceptor sites were detected in the 3' region of env. By hybridization analysis and sequencing of cDNA clones, it was revealed that the medium sized mRNAs are derived from a single splice event, with different splice acceptor sites, and that the two smaller transcripts are doubly or triply spliced mRNAs. Our results demonstrate a complex pattern of alternative splicing of FIV mRNAs. Furthermore, we identified monocistronic rev mRNA species that employ a unique splice acceptor site.

Introduction

The lentiviruses including human (HIV), simian (SIV), feline (FIV) and bovine immunodeficiency viruses (BIV), equine infectious anaemia virus, visna virus and caprine arthritis-encephalitis virus belong to the same family of retroviruses (for a review, see Narayan & Clements, 1990). These viruses cause slowly progressive multi-organ diseases in their natural hosts (Ishida et al., 1989; Narayan & Cork, 1985; Pedersen et al., 1987; Yamamoto et al., 1988). The genomes of the lentiviruses have been shown to encode several proteins that affect various aspects of viral expression or infectivity and are thought to play a role in these complex virus-host interactions (Cullen & Greene, 1989; Pavlakis & Felber, 1990). Levels of virus expression are determined to a great extent by the action of regulatory proteins.

The expression patterns of viral mRNAs have been well studied in HIV/SIV and visna virus systems (Davis et al., 1987; Schwartz et al., 1990; Viglianti et al., 1990). The primary full-length viral RNA transcript is processed into mRNAs by multiple splicing events and by a mechanism which appears to involve temporal regulation of gene expression (Cullen & Greene, 1989). The small, multiply spliced mRNA species, encoding the viral regulatory proteins Tat and Rev, are expressed in the early stage of infection. The tat gene product stimulates viral gene expression through its action on the long terminal repeat (LTR) and causes the accumulation of high levels of multiply spliced mRNA early in infection. The HIV-1 Rev activates the expression of late genes including the three viral structural genes (gag, pol and env), and the vif and vpr genes, whose mRNAs contain the cis-acting RNA target sequence for Rev designated the Rev-responsive element (RRE) (Garrett et al., 1991; Schwartz et al., 1991).

The genetic organization of FIV appears to be relatively simple compared with that of other lentiviruses in that the genome contains at least three short open reading frames (ORFs), vif, ORF-A and ORF-B, in addition to the structural genes common to all retroviruses (Maki et al., 1992; Olmsted et al., 1989; Talbott et al., 1989). Recently, we demonstrated that the vif gene of FIV is essential for cell-free virus infectivity, similar to the vif genes of various primate lentiviruses (Tomonaga et al., 1992). A short 3' ORF that overlaps the LTR, ORF-B, is at least part of rev gene (Kiyomasa et al., 1991; Phillips et al., 1992). The location of the FIV RRE was identified at the 3' end of the env ORF (Phillips et al., 1992), and not at the SU–TM junction (envelope surface protein–transmembrane protein), as in other lentiviruses.

The transcription patterns of several FIV mRNAs...
have recently been reported (Phillips et al., 1990, 1992; Tomonaga et al., 1992). However, the actual mapping of splice sites was not performed. Furthermore it is not known whether all transcripts are equally translated or whether a single protein is translated from mRNA that may encode sequences for more than one protein. In the present study, as one approach towards determining the products of the FIV genome that regulate its expression, we examined the expression pattern of FIV-specific RNAs and splice sites in an FIV-infected CD4+ lymphoblastoid cell line. The results revealed that FIV exhibited a complex splicing pattern, and that monocistronic rev mRNA in strain TM2 employed a unique splice acceptor site, which was not found in A. isoaltes.

**Methods**

**Cell culture and virus.** A feline CD4+ lymphoblastoid cell line (MYA-1 cells) (Miyazawa et al., 1989) was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). Crandell feline kidney (CRFK) cells (ATCC CCL94) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. MYA-1 cells infected with FIV strain TM2 (Miyazawa et al., 1989) were used for analysis by Northern (RNA) blot hybridization as described previously (Rabson et al., 1986). M59418.

**Transfection.** Uncleaved plasmid DNA was introduced into CRFK cells by the calcium phosphate coprecipitation method (Adachi et al., 1991) and FIV strain TM2-specific oligonucleotides were labelled with O'5-32P-labelled probes. The locations of the probes are: 2A, nt 5191 to 5290; VIA, nt 5316 to 5335; 4, nt 6264 to 6445; 5, nt 8912 to 9008.

**Cloning and sequencing of amplified cDNA.** After amplification of the cDNAs by PCR, the products were digested with appropriate restriction enzymes and cloned into pUC118 or pUC119 cloning vectors. For sequencing, the double-stranded plasmid DNAs were denatured and dissolved in the reaction mixture together with the sequencing primer. The DNA sequences were determined using the Sequenase version 2.0 kit (United States Biochemical).

**Analysis of PCR products.** To investigate the structure of the small, multiply spliced mRNAs of FIV, the PCR product amplified with primer pair PRLTS-PRREA was analysed on a 5% polyacrylamide gel. DNA was visualized by u.v. fluorescence after staining with ethidium bromide. To denature DNA before blotting, the gel was soaked in 0.5 M NaOH plus 0.6 M NaCl for 10 min, washed twice with H2O, and neutralized in 2 M Tris-HCl pH 7.0 plus 0.6 M NaCl for 10 min. The DNA was transferred to a nylon membrane by electroblotting at 35 mA for 16 h in 2xTBE buffer (0.1 M Tris-HCl pH 8.3, 0.1 M boric acid, 2.0 mM-EDTA) at 4 °C. After cross-linking of DNA, the membrane was prehybridized for 1 h at 37 °C. Hybridization was performed overnight with 32P-labelled probes. The locations of the hybridization probes are indicated in Fig. 8. The exact positions of the probes are: 2A, nt 5191 to 5290; VIA, nt 5316 to 5335; 4, nt 6264 to 6445; 5, nt 8912 to 9008.

**Results**

**Expression of viral mRNAs**

Previously we reported the transcription pattern of FIV by Northern blot analysis (Tomonaga et al., 1992). These studies revealed transcripts of 9.2, 5.2 and 4.4 kb which presumably represent gag-pol, vif and env mRNAs, respectively, as well as smaller transcripts (approximately 1.7 kb) which did not hybridize to vif and env probes (Tomonaga et al., 1992). To characterize these RNAs in more detail, we used a series of probes specific for different regions of the viral genome, and a TM2-specific oligonucleotide. The locations of each probe within the FIV genome are shown in Fig. 1.

The LTR probe (probe A in Fig. 1) clearly detected two distinct small, multiply spliced mRNAs, 1.7 and 1.4 kb, in addition to the genomic and medium sized mRNAs (Fig. 1, lane 1). The gag-pol probe (probe B in Fig. 1) detected only one genomic size mRNA, the 9.2 kb...
species (Fig. 1, lane 2). The specific probe for vif (probe C in Fig. 1) hybridized to the gag-pol and vif mRNAs (Fig. 1, lane 3). To identify the genetic content of the smaller mRNAs, a few small probes were used. Probe D, which does not contain a consensus splice acceptor site (Mount, 1982) just upstream from the env ORF, hybridized to the 9.2, 5.2, 4.4 and 1.7 kb RNA species (Fig. 1, lane 4). Probe E, which was the same oligonucleotide as primer PRENA located in the 5' end of env and did not contain the ORF-A sequence, detected 9.2, 5.2, 4.4, 1.7 and 1.4 kb RNA species (Fig. 1, lane 5). Probe F, which is located just downstream from a consensus splice donor sequence (Mount, 1982) in the 5' end of env, detected 9.2, 5.2, 4.4, 1.7 and 1.4 kb RNA species (Fig. 1, lane 6). The exact positions of the probes are: A, nt 16 to 515; B, nt 1751 to 2503; C, nt 5171 to 5936; D, nt 5936 to 6086; E, nt 6402 to 6421; F, nt 6694 to 7110; G, nt 7110 to 7957.

was supported by the analysis of splice sites (see below). The 4.4 kb transcripts are singly spliced and only the upper 4.4 kb species contains the ORF-A sequence. Probe G hybridized to genomic and all medium sized species (Fig. 1, lane 7). The ORF-B-specific probe detected all virus-specific bands, as did probe A (data not shown). In addition to these clear bands, less distinct bands were detected around 3.0 kb with the LTR (probe A), env (probe G) (Fig. 1, lanes 1 and 7), and ORF-B probes (data not shown).

Next, to investigate the role of the FIV Rev protein in expression of these transcripts, we analysed cytoplasmic poly(A)⁺ RNA from CRFK cells transfected with the rev⁻ mutant proviral clone, pTM-Nd (Tomonaga et al., 1992), by Northern blot analysis (Fig. 2). When the LTR-specific probe was used, all virus-specific bands were detected in the cells transfected with the wild-type viral clone, pTM219 (Fig. 2, lane 1). In contrast, the probe detected only small, multiply spliced mRNA species in the cells transfected with pTM-Nd (Fig. 2, lane 2). Compared with the wild-type, the 1.4 kb mRNA in the cell transfected with pTM-Nd was unclear, because the rev⁻ proviral clone was deleted between the two NdeI sites, which contained the splice acceptor site for the 1.4 kb transcript (see below). The env-specific probe hybridized to the genomic and medium sized mRNAs in the cells transfected with pTM219 (Fig. 2, lane 1). However, the probe failed to detect any specific mRNAs in the cells transfected with pTM-Nd (Fig. 2, lane 5). These results revealed that the cytoplasmic expression of the 9.2, 5.2 and 4.4 (doublet) kb mRNAs was dependent on the Rev protein.
Fig. 3. Detection of the transcription patterns of FIV by PCR. A schematic representation of the FIV genome is shown at the top. (a) Arrows indicate oligonucleotide primers used for cDNA synthesis. The exact positions of these oligonucleotides are indicated in Methods. (b) The locations of the splice donor (SD) and acceptor (SA) sites and structures of the viral mRNA transcripts deduced from results of hybridization to different probes and sequencing of partial cDNA clones. The corresponding nucleotide number of each splice site is indicated in the text.

Fig. 4. Splice junction of the 3′ region of the FIV genome. Schematic representation of part of the genome indicating the position of splice donor (SD3) and acceptor (SA4 and SA5) sites. The solid bars indicate the exons within the transcripts that employ splice sites SD3 and SA4 (a), and SD3 and SA5 (b). The black box in the ORF-B region indicates the sequence that encodes the Arg-Lys-rich domain of Rev.

Analysis of the splice junctions of FIV

To map splice junctions, partial cDNAs were constructed and amplified by using oligonucleotide primers. The primers used in this study are shown in Fig. 3(a). After amplification, the products were cloned into pUC118 or pUC119 vectors and sequenced to determine the splice donor and acceptor sites in the genome. To determine the splice sites, at least six clones were sequenced for each of the products. The 5′ splice donor site at nt 611 (SD1) of the TM2 sequence and the splice acceptor site at nt 5194 (SA1) (48 bases 5′ to the first AUG of the vif ORF) were previously reported (Tomonaga et al., 1992). The other splice sites were detected by combinations of each primer pair. One of the products amplified by using primer pair PRLTS–PROAA contained three exons. The second exon of the product employed the splice acceptor site for vif ORF (SA1), and terminated at the splice donor site at nt 5262 (SD2), and the third exon employed the splice acceptor site at nt 5928 (SA2) which was located close to the end of the vif ORF, 69 bases upstream of the ORF-A initiation codon.

Another product employed the splice donor (SD1) and acceptor (SA2), and consisted of only two exons. The splice acceptor site just 5′ of the env ORF at nt 6515 (SD3) was detected using primer pair PRLTS–PREAA and is predicted to be used for the generation of the env mRNA. The splice donor site which was located in the 5′ end of the env sequence and utilized for multiply spliced mRNA species was identified at nt 6515 (SD3). This splice donor site was joined to the splice acceptor site at either nt 8959 (SA4) or 9015 (SA5) (Fig. 4). Seventy-one percent of the clones obtained from the amplification by using primer pair PREAA–PRREA employed the splice donor site at nt 6515 (SD3) and the acceptor site at
nt 8959 (SA4). The splice acceptor site at nt 9015 (SA5) was located downstream of the conserved Arg–Lys-rich domain of the rev sequence of FIV (Fig. 4), suggesting that the rev mRNA of FIV TM2 employed the splice acceptor site at nt 8959 (SA4) but not at nt 9015 (SA5). We previously reported that the splice acceptor sites in the 3' portion of env were predicted to be at nt 8900 and 8959 (Maki et al., 1992); however, we did not detect use of the splice acceptor site at nt 8900 in this study.

Fig. 3 (b) shows the locations of splice sites on the FIV genome and the putative structure of each mRNA transcript which was deduced from the hybridization to different probes and sequencing of partial cDNA clones. The upper 4.4 kb mRNA was recognized by probe D (Fig. 1), suggesting that the transcript may employ the splice acceptor site at nt 5928 (SA2) and encode ORF-A and/or env products. The 1.7 kb mRNAs probably represent doubly or triply spliced mRNAs and were believed to be the ORF-A/rev mRNA. Transcripts similar to these two 1.7 kb mRNAs were also reported by Phillips and colleagues in the Petaluma isolate (clone 34TF10) (Phillips et al., 1992). The 1.4 kb mRNA may consist of the presumed glycoprotein leader sequence in the 5' region of the env ORF and 3' sequences of the genome. Recently, it has been reported that these two regions were essential for rev gene activity (Kiyomasa et al., 1991; Phillips et al., 1992). Thus, it is predicted that the transcript represents the monocistronic mRNA for the FIV rev gene.

To verify that the 1.4 kb transcript functions as a rev mRNA, we constructed the FIV Rev expression plasmid, pTM-Rev2.1, in which the SacI–Xbal fragment of plasmid pTM219, containing an intact provirus inserted into plasmid pUC19, was replaced by the SacI–Xbal fragment of a cDNA clone encoding the 1.4 kb transcript (Fig. 5a). The rev mutant (pTM-Nd) was transfected into CRFK cells with or without pTM-Rev2.1 and evaluated for transient expression of virion-associated RT. Although RT production was not observed in CRFK cells transfected with pTM-Nd, it was observed in CRFK cells cotransfected with pTM-Nd and pTM-Rev2.1, at wild-type levels (pTM219) (Fig. 5b). This result showed that pTM-Rev2.1 complements the rev- mutant, and hence that the 1.4 kb transcript functions as a messenger for the FIV rev gene. The sequence of monocistronic rev cDNA is shown in Fig. 6.
identical to those of the single intron in the env transcript, since they shared the same splice donor and acceptor sites for this region (Fig. 6).

Sequence analysis of the splice sites in FIV TM2 (Fig. 7a) revealed that nearly all splice sites were well conserved in the different FIV sequences. However, the sequence of the splice acceptor for env/rev mRNAs in FIV strain TM2 (SA3) was not conserved in American isolates (clones 34TF10 and PPR) (Phillips et al., 1992; Talbott et al., 1989), and we could not find analogous splice sites in their sequences (Fig. 7b).

From the results of the cDNA sequencing, the locations of the exons in the FIV genome were predicted. To identify the genomic regions within the FIV cDNAs, the cDNAs amplified with primer pair PRLTS-PRREA were transferred to nylon membranes and hybridized to different probes. The location of each probe and the results of hybridization are shown in Fig. 8. All bands visualized by ethidium bromide staining hybridized to the probe which corresponded to exon 1 (data not shown). Compared with probe VIA, probe 2A which encompassed non-coding exon 2A hybridized to many bands, strongly indicating that many multiply spliced mRNAs contained non-coding exon 2A. Probe VIA, which was located between exons 2A and 3, hybridized to only one band at above 1.3 kb, indicating that only one exon 2-containing mRNA was generated. Probe 4 was located in the overlap region of exons 3 and 4. Probe 5 did not contain exon 6 sequences. Thus, the bands detected with both probes 4 and 5 at between 0.64 kb and 0.47 kb may be mRNAs encoding the ORF-A and/or rev proteins. The product at below 0.47 kb was detected with probe 4, whereas probe 5 failed to detect it. This observation confirmed that the transcript using SD3 and SA5 was generated as shown in Fig. 4. The product at 0.34 kb hybridized only to probe 2A, suggesting the existence of at least one unidentified small exon in the genome. The smallest band was detected with probes 2A

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<th>NUCLEOTIDE POSITION</th>
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Fig. 7. Nucleotide sequences of the splice sites of FIV TM2 mRNA. (a) Splice donor and splice acceptor sites. The nucleotide sequence of various cDNA clones isolated by PCR amplification was determined. The nucleotide positions indicated refer to the positions of splice donor and splice acceptor sites. The splice donor and acceptor consensus sequences were obtained from a report by Mount (1982). (b) Nucleotide sequence of splice acceptor site for rev mRNA in TM2 compared with those of different isolates (clones 34TF10 and PPR). The nucleotide changes from the TM2 sequence are indicated by asterisks. Bars represent gaps introduced to optimize the alignment. The TAG codons in the box indicate the stop codons for ORF-A (or ORF-2). The ATG codons in the box indicate the initiation codons for rev-coding exon 1.
and 5 but not with probes VIA and 4. Based on its size and hybridization properties the transcript consists of the exons 1, 2A and 5.

**Discussion**

By Northern blot analysis, cDNA synthesis and DNA sequencing, our results demonstrate that the FIV strain TM2 in the feline CD4+ lymphoblastoid cell line has a complex pattern of viral gene expression employing alternative splicing strategies similar to those observed in other lentivirus-infected cells (Davis et al., 1987; Noiman et al., 1990; Oberste et al., 1991; Schwartz et al., 1990; Viglianti et al., 1990).

In addition to the 9.2 kb genome length RNA, five other distinct viral RNA species were detected. The cytoplasmic expression of these transcripts was examined with the rev- proviral clone (Fig. 2). The rev mutant expressed only small, multiply spliced mRNAs but not unspliced or singly spliced mRNAs, 9.2, 5.2 and 4.4 (doublet) kb species in the cytoplasm. This result clearly indicated that the cytoplasmic expression of the 9.2, 5.2 and 4.4 (doublet) kb mRNAs was Rev-dependent and that these transcripts were expressed at late stages of the FIV replication cycle in infected cells.

All subgenomic RNAs contain a short 5' leader spliced to different regions of the genome 3' of the pol gene. It was found that the splice donor site at nt 611 (SD1) was utilized for all subgenomic RNA species. The two mRNAs of 4.4 kb have different genetic structures; the larger band contains sequences from ORF-A, whereas the smaller species contains the env ORF. Two splice acceptor sites are present in this region (at nt 5928 and 6264) and are probably utilized in these mRNAs. Either or both of these transcripts could function as the env mRNA. However the larger transcript contains the entire ORF-A and thus may function to produce a protein product from this region.

The 1.7 and 1.4 kb transcripts are at least doubly spliced, containing sequences from the 5' end of the genome spliced to sequences located between pol and env which are then spliced to sequences 3' of the env ORF. The 1.7 kb transcripts contain sequences from the whole of ORF-A spliced to sequences in ORF-B. These transcripts may have a bicistronic coding capacity and function as ORF-A and rev mRNAs. The finding of these 1.7 kb transcripts was supported by the recent report of Phillips et al. (1992). The 1.4 kb transcript was the monocistronic rev mRNA and the first coding exon overlapped and was in the same reading frame as the env ORF (Fig. 6). It is a unique finding that the splice acceptor site for monocistronic rev mRNA (SA3) in strain TM2 is not conserved in other strains (Fig. 7b), in contrast to other splice sites which are well conserved. In fact, only bicistronic mRNAs (ORF-A and rev) have been identified in American isolates. Schwartz et al. (1990) reported that HIV tat mRNAs produced very low levels of Rev compared to mRNAs transcribed from only rev. Although FIV ORF-A has an unknown function, assessment of the relative translation efficiency of the bicistronic and monocistronic mRNAs will be important for a full understanding of the regulation of virus gene expression.

In our study, two splice acceptor sites (SA4 and SA5) were detected in the 3' region of env, whereas Phillips et al. (1992) found only one (SA4 equivalent) in this position. The transcripts employing SA5 were minor species and could not form rev mRNA. However it is possible that this splice acceptor site is employed for the last exon of the mRNA exclusive to ORF-A, or another mRNA which encodes a novel protein. Our Northern blot analysis using LTR and gene-specific probes revealed that the mRNAs of approximately 3.0 kb hybridize with the LTR (probe A), env (probe G) or ORF-B probes, indicating that these mRNAs contain the exon that is located in the env ORF, and as such are similar to tev in HIV-1 transcripts (Benko et al., 1990; Salfeld et al., 1990). Further study is needed to determine the identity of these RNA species.

A non-coding exon, 2A, is located in the overlap region of the pol and vif genes (Fig. 8). The exon is
conserved in another strain of FIV (Phillips et al., 1992) as a leader sequence of transcripts. Many cDNAs hybridized to the probe that encompassed exon 2A, suggesting that this non-coding exon was contained in many multiply spliced mRNA species. Although it is not clear whether the length of the leader sequence has any effect on the function of the transcripts, it is noteworthy that the mRNAs containing exon sequences similar to 2A in FIV are produced by other lentiviruses such as HIV-1, the macaque strain of SIV and visna virus (Davis & Clements, 1989; Sodroski et al., 1985; Viglianti et al., 1990). An mRNA containing exon 2 was detected by hybridization (Fig. 8). This mRNA contains the entire vif sequence and may be the FIV equivalent of the HIV-1 mRNA expressing the Vif protein, which is doubly spliced (Arya & Gallo, 1986). From the results of the experiment shown in Fig. 8, it is predicted that more transcripts might be generated in the infected cells than we could detect.

Our study and previous reports of others demonstrate that lentiviruses including FIV share a pattern of mRNA expression with complex alternative splicing, suggesting that the expression of lentivirus genomes may be controlled at many different levels. Therefore, further extensive studies will be important for our understanding of the viral life cycle and the significance of viral gene regulation in pathogenesis.

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References


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