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Identification of the Rev Transactivation and Rev-Responsive Elements of Feline Immunodeficiency Virus

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Spliced messages encoded by two distinct strains of feline immunodeficiency virus (FIV) were identified. Two of the cDNA clones represented mRNAs with bicistronic capacity. The first coding exon contained a short open reading frame (orf) of unknown function, designated orf 2. After a translational stop, this exon contained the L region of the *env* orf. The L region resides 5′ to the predicted leader sequence of *env*. The second coding exon contained the H orf, which began 3′ to *env* and extended into the U_3 region of the long terminal repeat. The in-frame splicing of the L and H orfs created the FIV *rev* gene. Site-directed antibodies to the L orf recognized a 23-kDa protein in infected cells. Immunofluorescence studies localized Rev to the nucleoli of infected cells. The Rev-responsive element (RRE) of FIV was initially identified by computer analysis. Three independent isolates of FIV were searched in their entirety for regions with unusual RNA-folding properties. An unusual RNA-folding region was not found at the Su-TM junction but instead was located at the end of *env*. Minimal-energy foldings of this region revealed a structure that was highly conserved among the three isolates. Transient expression assays demonstrated that both the Rev and RRE components of FIV were necessary for efficient reporter gene expression. Cells stably transfected with *rev*-deleted proviruses produced virion-associated reverse transcriptase activity only when FIV Rev was supplied in *trans*. Thus, FIV is dependent on a fully functional Rev protein and an RRE for productive infection.

In addition to the genes common to all retroviruses (*gag*, *pol*, and *env*), lentiviruses also encode regulatory genes (5–7, 9, 16, 28, 38). These regulatory genes are expressed from multiply spliced mRNAs which contain the small open reading frames (orfs) from the intergenic region spliced to segments from the 3′ end of the genome. The virus-encoded regulatory proteins orchestrate the expression of the various lentiviral proteins (5, 6, 9, 16, 38, 42). Rev is a regulatory protein that has a pivotal role in determining whether an infection is latent or productive (27). When the cell concentration of Rev is high, most of the viral mRNA in the cytoplasm is full length or singly spliced. When the concentration of Rev is low, most of the cytoplasmic mRNA is small and multiply spliced (8, 10, 11, 18). Full-length or singly spliced mRNA codes for virion proteins Gag, Pol, and Env. Multiply spliced mRNAs code for regulatory factors Rev, Tat, and Nef. Thus, high cellular concentrations of Rev result in virus production and low Rev concentrations maintain the virus in a latent state. Feline immunodeficiency virus (FIV), a recently isolated lentivirus of domestic cats (32), has the coding capacity for similar regulatory genes (33, 39). However, neither the regulatory genes nor their products have been fully characterized for FIV. The purpose of this study was to identify the Rev protein and Rev-responsive element (RRE) of FIV.

The genomic organization of FIV is similar to that of visna virus. In visna virus and FIV, a region of the *env* orf termed L is located 5′ to the presumed glycoprotein leader sequence (6, 33, 39). In visna virus, the L domain is the first exon of *rev* (40). By analogy, the L domain of FIV may have a similar function. Recently, FIV Rev activity was demonstrated by analyzing the viral genome with genetically engineered deletions (17). FIV Rev activity roughly mapped to three areas: (i) the 5′ splice donor site, (ii) the region spanning from the 3′ end of *pol* through the 5′ end of *env*, and (iii) the H orf at the 3′ end of the genome (17).

Rev mediates its effects by binding to an RNA higher-order structural element designated the RRE (22, 24, 31). In all of the examined lentiviruses, the RRE is located near the Su-TM junction of the *env* orf (1, 4, 22, 34, 35, 40, 41). The RRE of FIV was previously shown to map to a 1,000-bp fragment of *env* that encompasses the Su-TM junction and extends past the *env* termination codon (17).

In the present study, we identified a cDNA that encodes FIV Rev and mapped the RRE to a 243-bp fragment at the 3′ end of the *env* gene. FIV Rev was translated from a gene that arose by in-frame splicing of the L domain of *env* to the H orf. The RRE of FIV was found not at the Su-TM junction, as in other lentiviruses, but rather at the 3′ end of the *env* orf. Both the FIV Rev and RRE components were necessary for reporter gene expression.

MATERIALS AND METHODS

Cells and viruses. 34TF10, an infectious molecular clone of the Petaluma isolate of FIV, was transfected into Crandall feline kidney (CrFK) cells as previously described (39). An interleukin 2-independent feline lymphocyte line that contin-

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uously expresses the PPR clone (33) was kindly provided by E. Sparger. The 34TF10 and PPR clones have different cell tropisms, vary by 15% in *env*, and originate from geographically diverse regions (33).

cDNA preparation. Ten days following transfection with the 34TF10 clone, 10^8 CrFK cells were harvested for mRNA, as were 5×10^8 lymphocytes that continuously express the FIV PPR clone. mRNA was prepared by using an oligo(dT) column system (Invitrogen Fast Track). The cDNA was made by conventional methods (37), by using a specific primer located in a conserved area of the R region of the long terminal repeat at bases 9353 to 9376 of the PPR clone (33) and 9357 to 9380 of the 34TF10 clone (39).

PCR amplification and cloning of the cDNA. Amplification of the cDNA was accomplished by using 5′ and 3′ polymerase chain reaction (PCR) primers that included *Eco*RI restriction enzyme sites. The 5′ primers were located in a highly conserved region of U_5 (bases 312 to 341 for PPR [33], and bases 313 to 342 for 34TF10 [39]). The 3′ PCR primers were conserved sequences in the U_3 region of the long terminal repeat at bases 9207 to 9232 of the PPR clone and bases 9211 to 9236 of the 34TF10 clone. After 40 cycles of amplification (2 min of denaturation at 94°C, 2 min of annealing at 54°C, and 2 min of extension at 72°C), the reaction product was extracted with phenol-chloroform, ethanol precipitated, digested with *Eco*RI, and purified by agarose gel electrophoresis. The resulting bands were ligated into M13, and sequencing was performed by the dideoxy-chain termination method as previously described (36).

In vitro transcription and translation. All of FIV PPR cDNA clone 2 (see Fig. 2) was subcloned into BlueScript (KS−) and transcribed and translated in vitro by using T7 and T3 RNA polymerases and a rabbit reticulocyte lysate translation system (Promega).

Production of antipeptide antibody. A peptide (RQWIGPEEAEELLDF) derived from the predicted amino acid sequence of coding exon 1 of *rev* (L) was synthesized as previously described (29). The peptide was coupled to keyhole limpet hemocyanin via an artificial cysteine added to the C terminal as previously described (21) and inoculated into rabbits for production of antisera.

Western immunoblot analysis. Proteins from FIV-infected cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nylon membranes. The blot was blocked with BLOTTO (15) overnight at 4°C and then incubated overnight at 4°C with rabbit antipeptide sera in the presence or absence of cognate peptide to validate specificity. The blots were then washed with BLOTTO and incubated for 3 h at 22°C with goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate. Unbound secondary antibody was then removed by washing with two changes of BLOTTO, followed by a 30-min wash with 0.5 M LiCl–1% Nonidet P-40. Bound antibodies were detected by the chemiluminescence reaction (ECL; Amersham).

Indirect immunofluorescence. Chronically infected feline lymphocytes were cytospun, acetone fixed for 5 min, and washed with phosphate-buffered saline. The cells were incubated at room temperature with rabbit antipeptide sera for 30 min, washed, incubated for 30 min with goat anti-rabbit immunoglobulin G fluorescein conjugate, washed, counterstained with methylene blue, and examined for fluorescence.

Detection of unusual RNA-folding regions (UFRs). Two criteria were used to select regions of interest. (i) The free energy (*e*) was significantly lower than the mean free energy of a large sample of randomized sequences with the same base composition and same length (*er*) [expressed as significance score = $(e - er)/sdr$, where *sdr* was the standard deviation of the random sample]. (ii) The free energy (*e*) was relatively low compared to the mean of the free energies of all other segments of the same length in the biological sequence under consideration (*eb*) [expressed as stability score = $(e - eb)/sdb$, where *sdb* is the standard deviation of the sample]. Thus, segments were considered significant when they had both a significant score less than −2.0 and a stability score among the lowest 5% within the genome (19). By using a window size ranging from 50 to 200 nucleotides, the entire genomes of the three molecular clones of FIV (34TF10 [39], TM2 [17], and PPR [33]) were searched.

Minimal-energy foldings. Optimal folding and suboptimal foldings were calculated through minimization of free energy by the algorithm described by Zuker (43). Analysis was performed with the free-energy values given by Jaeger et al. (14). For the FIV 34TF10, TM2, and PPR sequences, a set of suboptimal foldings were generated with different window sizes (ranging from 0 to 10) and within a 10% energy difference from the optimal structure.

Chloramphenicol acetyltransferase (CAT) reporter gene expression. The spliced gene (L to H) from PPR cDNA clone 2 was subcloned into simian virus 40 expression plasmid p24A7 (supplied by Peter Barry) and designated pRev (see Fig. 7). An infectious molecular clone, FIV 34TF10, was also used as a source of FIV Rev (39). The CAT reporter plasmids were modeled after pDM128 (13). pCAT expressed the CAT gene, driven by the Rous sarcoma virus long terminal repeat promoter. For pRRE(+), a 243-bp fragment from FIV PPR (33) (*Eco*RI, base 8647, through *Stu*I, base 8990) was inserted downstream of the CAT gene in pCAT. This fragment contained the predicted RRE of FIV PPR in the sense orientation. Flanking the CAT gene and the FIV RRE were splice donor and acceptor sites. pRRE(−) was identical to pRRE(+), except that the FIV RRE was cloned into the plasmid in the antisense orientation (see Fig. 7). The plasmids were transfected into CrFK cells by using DEAE-dextran as previously described (2). Following transfection, the cells were incubated for 48 h, harvested, and assayed for CAT activity by the protocol of Nordeen and colleagues (30).

***Trans* complementation of Rev-deleted FIVs.** The Δ*rev* provirus was constructed by deleting the splice acceptor site and the basic amino acid domain of the second coding *rev* exon from the FIV 34TF10 molecular clone. The 34TF10 clone has a unique *Asu*II site located at base 8920. The PCR was used to amplify DNA from base 8900 of the 34TF10 clone to the *Sal*I site of the polylinker. The 5′ PCR primer was modified to introduce an *Asu*II site at base 9020. The Δ*rev* provirus was produced by cloning the PCR-generated DNA into the *Asu*II and *Sal*I sites of the 34TF10 clone, resulting in a 100-bp deletion. The construct was confirmed by nucleotide sequence analysis. Three Δ*rev* provirus clones, 1 to 3, were obtained and stably transfected into CrFK cells by cotransfection with plasmid pRc/CMV (Invitrogen), which encodes neomycin resistance.

By using the PCR to generate the appropriate restriction sites, the *rev* gene of FIV PPR was cloned into bacterial expression plasmid pRSETB (Invitrogen) at the *Bam*HI and *Eco*RI sites. The FIV Rev protein was detected in bacterial cell lysates by Western blot analysis (data not shown). Bacterial concentrations were determined by optical densities. Protoplasts were prepared from Rev-expressing and nonexpressing *Escherichia coli* and fused to either control CrFK cells or the stably transfected Δ*rev* cell lines (clones 1

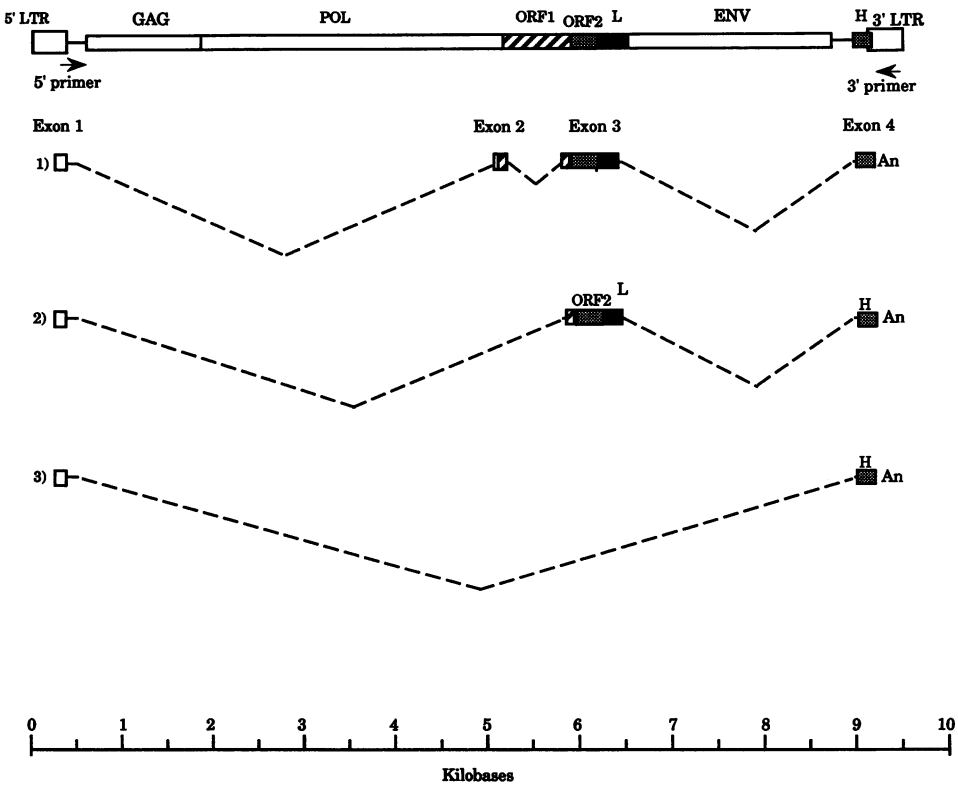


FIG. 1. Map of FIV cDNA clones. At the top is a diagrammatic representation of the orfs of FIV. The splicing patterns and orfs of all three cDNA clones are indicated. The dotted lines represent introns, and the rectangles represent exons. Arrows represent the approximate locations of the 5' and 3' PCR primers. "An" indicates the location of the presumed poly(A) tail. LTR, long terminal repeat.

to 3) by procedures previously described (26). At 36 h postfusion, the cell supernatants were monitored for reverse transcriptase (RT) activity as previously described (39).

RESULTS

Three subgenomic cDNA clones were identified from CrFK cells infected with either FIV 34TF10 or FIV PPR (Fig. 1). Clones 2 and 3 were recovered from both chronically FIV PPR-infected lymphocytes and 34TF10-infected CrFK cells. However, clone 1 was obtained only from 34TF10-infected CrFK cells. All three clones shared a common exon 1, ending with the previously identified splice donor site downstream of the 5' long terminal repeat (33). cDNA clone 1 had three additional exons. Exon 2 of this cDNA was approximately 70 bp long and included the start codon orf 1, the putative *vif* gene. This reading frame was not maintained upon splicing, owing to the introduction of a stop codon. Exon 3 contained orf 2 and the L region of the *env* orf. Exon 4 contained orf H and was spliced in frame to L. Splicing of L to H created the predicted *rev* gene of FIV. cDNA clone 2 was identical to cDNA clone 1, except that clone 2 lacked noncoding exon 2. cDNA clone 3 contained only exons 1 and 4. All splice donor and acceptor sites were conserved between the two infectious molecular clones, except for the last acceptor site. In the PPR clone, this acceptor site was 6 bases 5' to the corresponding site in the 34TF10 clone. Thus, the exon 2 of *rev* in PPR was two amino acids longer than the corresponding exon in the 34TF10 clone. cDNA clones 1 and 2 had a bicistronic coding capacity, as well as upstream AUGs prior to orf 2 and the putative *rev* gene (see the sequence of clone 2 in Fig. 2). The translational start of *rev* was located the farthest downstream but was in a favorable context for translation initiation (18). Additionally, a strong basic amino acid domain, typical of other lentiviral *rev*-encoded proteins (22, 23, 31), was observed near the 5' end of orf H (Fig. 2).

Because of the upstream translational starts, it was essential to show that the cDNA clones encode a protein that was actually translated. Thus, cDNA clone 2 from FIV PPR (Fig. 2) was selected for in vitro transcription and translation. The sense mRNA of clone 2 produced a 23-kDa protein (data not shown). This protein was not present in the antisense translation mixture. Furthermore, the 23-kDa protein was immunoprecipitated by a site-directed antibody specific for a portion of the predicted amino acid sequence of the first coding exon of *rev* (data not shown). Although the cDNA had a bicistronic coding capacity, a protein corresponding in size to the predicted orf 2 product was not detected.

To demonstrate the presence of the 23-kDa protein in FIV-infected cells, Western blot analysis was performed. The protein was not found in uninfected CrFK cells (Fig. 3, lane 1). However, a strong band of approximately 23 kDa was detect in FIV-infected CrFK cells (Fig. 3, lane 2). The specificity of the antipeptide sera for the 23-kDa protein was demonstrated by the inability of the sera to react with the 23-kDa band in the presence of the cognate peptide (Fig. 3, lane 4). Using the antipeptide antibody, immunofluorescence localized the 23-kDa protein to the nucleoli of FIV PPR-infected lymphocytes (Fig. 4).

To identify potential RNA secondary structures that might serve as an RRE, the entire genomes of three clones of FIV

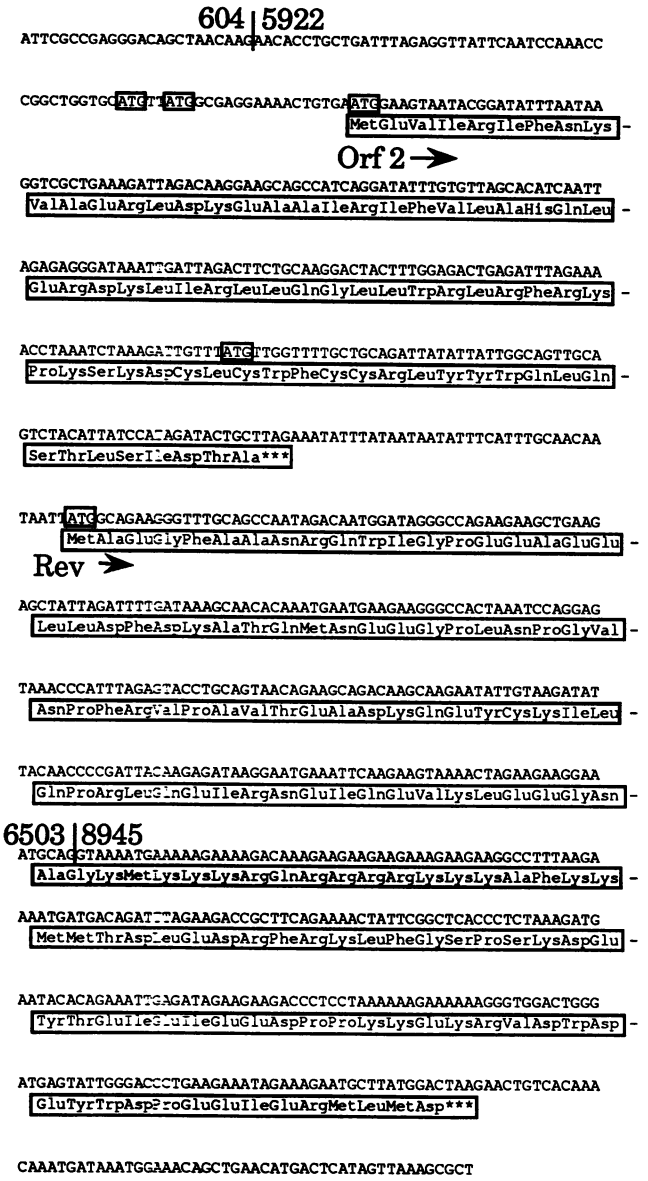


FIG. 2. Sequence and organization of clone 2. The large numbers separated by single vertical lines indicate exon junctions. Potential translation initiation codons of the RNA are boxed.

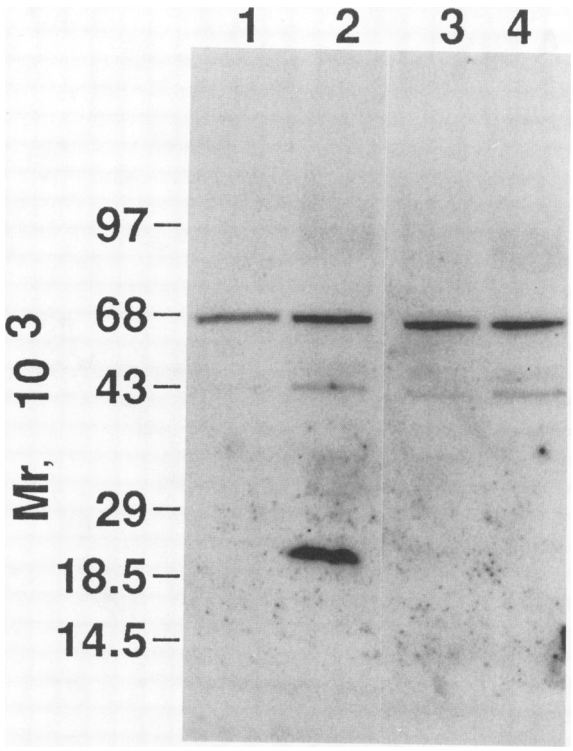


FIG. 3. Western blot analysis of lysates from FIV-infected cells. Lanes 1 and 3 are lysates of noninfected CrFK cells. FIV-infected CrFK cells lysates are shown in lanes 2 and 4. All of the strips were reacted with a rabbit antipeptide (L) serum made to the a peptide region from coding exon 1 of Rev (see Materials and Methods). However, strips 3 and 4 were incubated with antiserum that was absorbed with the cognate peptide. Note the detection of the 23-kDa band only in virus-infected cells, lane 2, and the inability of the cognate peptide-treated serum to bind to this protein (lane 4).

(PPR, 34TF10, and TM2) were first examined for unusual UFRs in terms of free-energy properties (see Materials and Methods). This search was similar to those performed previously on other lentiviruses that revealed highly significant UFRs near the Su-TM junction of *env* (20, 22, 35). These UFRs were eventually demonstrated to correspond to the RRE regions of these viruses. None of the three FIV sequences had a UFR around the Su-TM junction of *env* (for example, see Fig. 5; the location of the Su-TM junction is base 8,089). Comparison of optimal and suboptimal free-energy structures of the FIV sequences around the Su-TM junction also showed no potential structural conservation among the FIV clones (data not shown). However, the analysis revealed a common and particularly outstanding UFR within all FIV sequences. This UFR spanned the *env* stop codon and mapped to an optimal length of approximately 150 nucleotides (Fig. 5, bases 8776 to 8922). Minimal-energy folding of this region showed an extended single-hairpin structure with loop regions along the stem. This secondary structure is highly conserved among the 34TF10, TM2, and PPR clones (see Fig. 7). Among the three sets of optimal and considered suboptimal foldings, the three optimal foldings showed the closest similarity in structure. Furthermore, the suboptimal foldings of the three sequences show no relevant alternative structures (i.e., they showed only local and minor variations). Several nucleotide differences among the three FIV sequences were recognized (Fig. 6A and B). The differences either occurred in the predicted loops or were changes that maintained the base pairing of the calculated minimal-energy structure. These compensatory changes support the functional importance of the proposed RNA structure and suggest a possible RRE role.

On the basis of these findings, we constructed reporter plasmids that contained the FIV RRE in the positive-sense and antisense orientations downstream of the CAT reporter gene in the vector pCAT. In these reporter constructs, the RREs and CAT genes were flanked by splice donor and acceptor sites (Fig. 7A), so that splicing would remove the CAT gene from the transcript. Similar reporter constructs were used by others to study Rev function in the human immunodeficiency virus (13) and FIV (17) systems. Splicing was then monitored in the presence or absence of Rev. The CAT levels were monitored in the presence or absence of

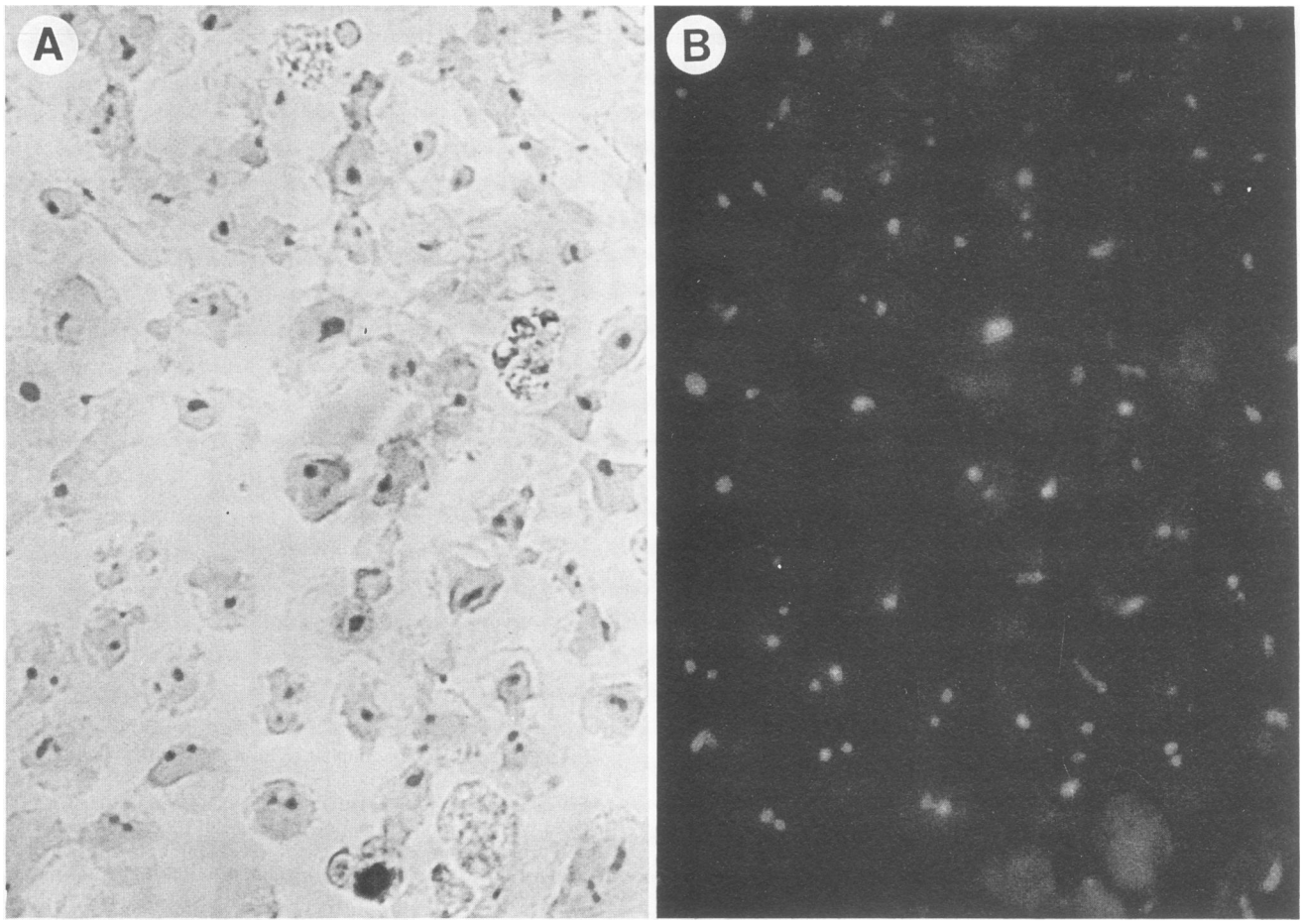


FIG. 4. Localization of FIV Rev. Indirect immunofluorescence on FIV-infected cells using an antipeptide antibody made to exon 1 of Rev. Panels A and B show the same field. However, panel A was taken under normal microscopy, whereas panel B was taken under fluorescence microscopy. Note that the methylene blue in panel A and the immunofluorescence in panel B both stained the nucleolus.

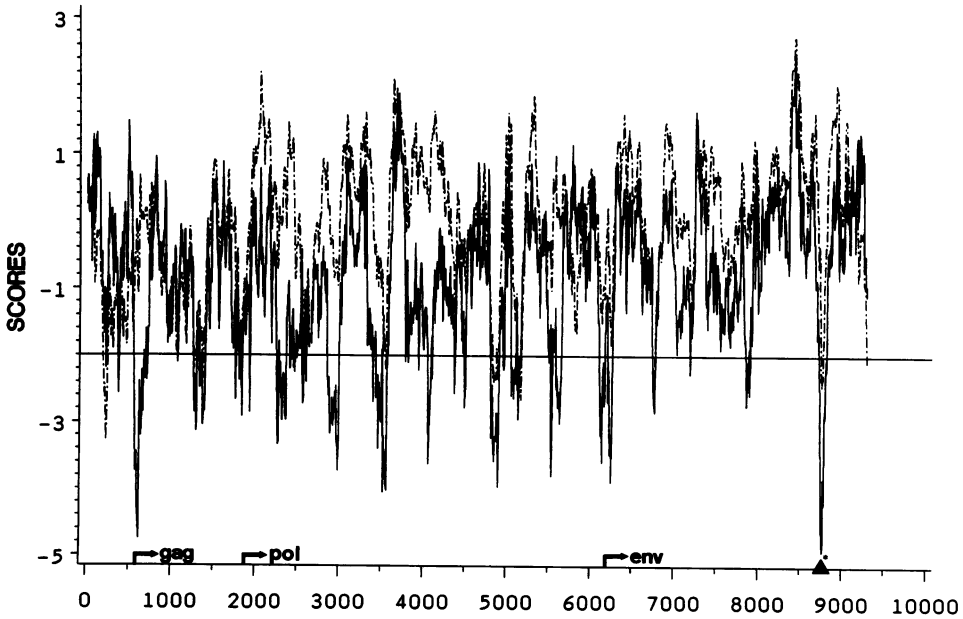


FIG. 5. Distribution of the significance and stability scores over the FIV PPR genome. The map presented was generated with a segment size of 150 nucleotides. The significance score (solid line) and the relative stability score (broken line) are plotted against their genomic locations. Scores have been averaged in groups of five positions. The translational starts of the *gag*, *pol*, and *env* genes are indicated. The *env* stop codon is depicted by the asterisk. The highly significant UFR around the *env* stop codon is marked by an arrowhead (bases 8776 to 8922). The predicted Su-TM junction of FIV is located at approximately base 8,089.

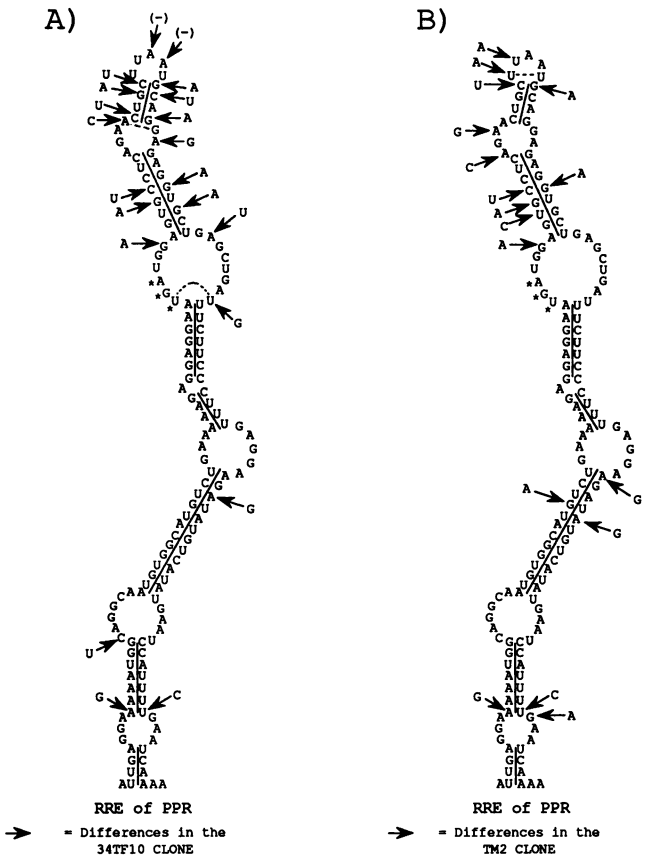
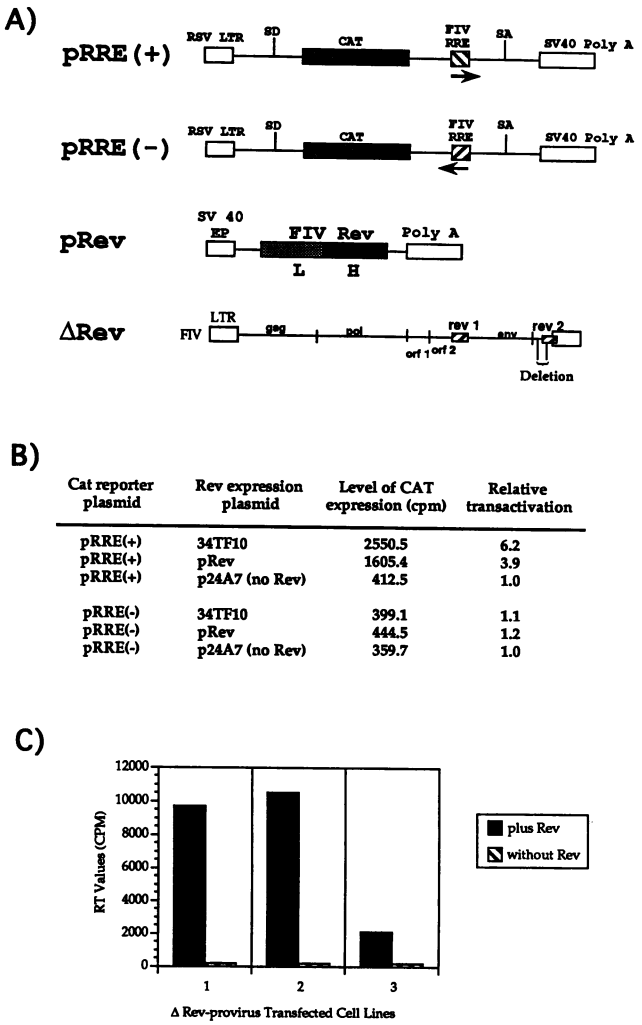


FIG. 6. Calculated secondary RNA structure of the FIV RRE. The structure is based on minimal-energy folding of the 150-nucleotide UFR around the *env* stop codon (Fig. 5). The secondary RNA structure of the FIV PPR clone and the locations of nucleotide changes of the 34TF10 clone are shown in part A. Part B depicts the same PPR RRE structure. However, the nucleotide changes indicated in part B are for the TM2 clone. The nucleotide changes from the PPR sequence are denoted by arrows. The symbol (−) represents a nucleic acid deletion at that location in the 34TF10 clone. The asterisks indicate the *env* translational stop codon.



Cat reporter plasmid	Rev expression plasmid	Level of CAT expression (cpm)	Relative transactivation
pRRE(+)	34TF10	2550.5	6.2
pRRE(+)	pRev	1605.4	3.9
pRRE(+)	p24A7 (no Rev)	412.5	1.0
pRRE(-)	34TF10	399.1	1.1
pRRE(-)	pRev	444.5	1.2
pRRE(-)	p24A7 (no Rev)	359.7	1.0

FIG. 7. Assays for Rev activity. (A) Diagrammatic representation of the CAT reporter gene constructs, the Rev expression vector, and the FIV Δ*rev* provirus. pRRE(+) contains the CAT gene and the FIV RRE in the sense orientation, which are flanked by splice donor (SD) and acceptor (SA) sites. pRRE(−) is the same as pRRE(+), except that the FIV RRE is inserted in the antisense orientation. pRev is designed for eukaryotic expression of FIV Rev. Δ*rev* is a deletion mutant of the infectious FIV 34TF10 molecular clone. SV40, simian virus 40; RSV, Rous sarcoma virus; LTR, long terminal repeat. (B) Relative transactivation of FIV Rev in CrFK cells. Cells were cotransfected with a reporter plasmid and the Rev expression plasmid or p24A7 (vector). Relative transactivation was calculated with respect to p24A7. (C) *trans* complementation of the Δ*rev* provirus by bacterially expressed FIV Rev. Bacterial protoplasts from either *rev*-expressing (plus Rev) or non-*rev*-expressing (without Rev) *E. coli* were separately fused with CrFK cells and the three Δ*rev* provirus cell lines, 1, 2, and 3. The RT values represent the total RT value minus the RT value of CrFK cells fused with either *rev*-expressing or -nonexpressing *E. coli*.

FIV Rev, which was supplied either by transfection with an infectious molecular clone (34TF10) or by expression plasmid pRev, which contains the L and H regions of cDNA clone 2 of FIV PPR (Fig. 7A). CrFK cells transfected with pCAT, lacking the RRE, showed low levels of CAT activity in the presence or absence of pRev (data not shown). CrFK cells transfected with pRRE(−), in the presence or absence of pRev or the infectious FIV molecular clone, also expressed low levels of CAT (Fig. 7B). However, CrFK cells cotransfected with pRRE(+) and either 34TF10 or pRev had high CAT levels (Fig. 7B). These findings demonstrate that expression of CAT in this reporter system was dependent upon the correct orientation of the RRE in the CAT reporter plasmid and coexpression of the Rev protein. These data are compatible with the interpretations that the RNA secondary structure identified by computer analysis is the RRE of FIV and that the 23-kDa protein arising from the gene created by the in-frame splicing of L to H is the Rev component of FIV.

To determine whether the FIV Rev protein also regulates virus protein expression, Δ*rev* constructs were prepared (Fig. 7A), cloned, and stably transfected into CrFK cells. No significant levels of particle-associated RT activity were detected in these cell lines. Bacterial protoplasts from either Rev-expressing or -nonexpressing *E. coli* were separately fused with each of the Δ*rev* cell lines (clones 1 to 3), as well as control CRFK cells. When fused with the FIV *rev*-expressing bacteria, all of the stably transfected cell lines gave markedly elevated particle-associated RT activity (Fig. 7C). Fusions with *E. coli* protoplasts not producing FIV Rev failed to elicit an increase in RT levels. These results

demonstrate a dependence on functional Rev for productive FIV infection.

DISCUSSION

We have identified the Rev and RRE components of FIV. The locations of these components are consistent with the approximate locations reported by Kiyomasu and colleagues (17). In addition to the 5′ splice donor site, they mapped FIV Rev activity, by introducing mutations into the genome, to two areas: (i) the intergenic region from 3′ end of *pol* through the L region of the *env* orf, and (ii) the H orf located at the 3′ end of the virus (17). Kiyomasu et al. also mapped the RRE to a 1,000-bp fragment containing both the Su-TM junction and the 3′ end of the *env* gene (17). In the present study, it was shown that the *rev* gene was created by in-frame splicing of L to H. Computer analyses revealed an RRE 150 bp long located at the end of the *env* gene. Transient expression assays demonstrated that both the RRE (in an orientation-dependent manner) and Rev of FIV were required for efficient CAT reporter gene expression.

The organization of the 5′ *env* orf of FIV is comparable to that of visna virus. In both viruses, the L domain of the *env* orf was demonstrated to encode the first exon of Rev (40). In each virus, this L domain precedes the presumed hydrophobic leader sequence of Env. The role of the L region in the Env precursor is not known. However, at least for FIV, it is likely that the L domain is part of the Env precursor and is removed by posttranslational cleavage prior to or along with the Env leader sequence. The second exon of FIV Rev began 3′ to the end of *env*. This location is further downstream in FIV than in other lentiviruses.

Although divergent at the amino acid level, FIV Rev was similar to the Rev components of other lentiviruses, having a highly basic amino acid domain and a nucleolar location. This basic domain in other lentiviruses is responsible for binding to the RRE and targeting of Rev to the nucleolus of the cell (23, 24, 31). Thus, it seems likely that the basic domain of FIV Rev has a similar function. The leucine-rich activation domain has been identified in human immunodeficiency virus type 1 and visna virus (23, 31, 41). Presumably, this domain is required for interactions of Rev with cellular factors necessary for transportation of viral transcripts out of the nucleus or prevention of splicing. Although the activation domains in human immunodeficiency virus type 1 and visna virus are conserved and interchangeable (41), strong amino acid homology with the activation domain of FIV Rev is not apparent. This lack of homology may mean that the activation domain of FIV Rev interacts with a different cellular factor or mediates its effect by another mechanism.

At the structural level, all of the RREs share a common feature: a long closing stem composed of several perfect helical elements interrupted by loops. The long stem structure may expose the active sites for intermolecular interactions or act as a scaffolding on which these reactions can occur. The exact association of the RRE with the various other elements involved in splicing awaits further elucidation.

Computer analysis showed that RRE of FIV is located at the end of the *env* gene. In the other members of the lentivirus subfamily, the RRE is located around the Su-TM junction of the *env* gene (1, 4, 22, 34, 35). In transient expression assays, the RRE of human immunodeficiency virus type 1 was shown to function at the Su-TM junction and in several other positions relative to the reporter gene (25). However, the position of the RRE may be important at the genomic level for coordination of viral gene expression. The location of the RRE and exon 2 of Rev might be linked. Exons 2 of Rev and the RRE are both located more 3′ in FIV than in other lentiviruses. The location of these elements in FIV suggests that a certain spatial arrangement must be maintained between the RRE and the splice acceptor site of exon 2 of Rev. In the process of binding to the RRE, Rev forms a riboprotein complex that extends along the mRNA in both directions (12, 24, 31). As the Rev riboprotein complex nears the splice acceptor site of Rev exon 2, it may interfere with spliceosome formation (3, 12). Thus, the effects of Rev on viral gene expression may depend, in part, on the distance between the RRE and Rev exon 2. Although highly speculative, this theory of Rev action is consistent with the organization of the FIV genome and may explain the unusual location of the FIV RRE. The findings of this study underscore the value of examining the diverse members of the lentivirus family to establish insights into the functions of the group as a whole.

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REFERENCES

1. **Aepinus, C., R. Voll, M. Broker, and B. Fleckenstein.** 1990. A rev/beta-galactosidase fusion protein binds in vitro transcripts spanning the rev-responsive element of human immunodeficiency virus type 1 (HIV-1). FEBS Lett. **263**:217–221.
2. **Barry, P. A., E. Pratt-Lowe, B. M. Peterlin, and P. A. Luciw.** 1990. Cytomegalovirus activates transcription directed by the long terminal repeat of human immunodeficiency virus type 1. J. Virol. **64**:2932–2940.
3. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of splice sites. Cell **59**:789–795.
4. **Cheng, S. M., M. Blume, S. G. Lee, P. P. Hung, V. M. Hirsch, and P. R. Johnson.** 1990. The simian immunodeficiency virus (SIV) rev gene regulates env expression. J. Med. Primatol. **19**:167–176.
5. **Cullen, B. R.** 1991. Human immunodeficiency virus as a prototypic complex retrovirus. J. Virol. **65**:1053–1056.
6. **Davis, J. L., and J. E. Clements.** 1989. Characterization of a cDNA clone encoding the visna virus transactivating protein. Proc. Natl. Acad. Sci. USA **86**:414–418.
7. **Davis, J. L., S. Molineaux, and J. E. Clements.** 1987. Visna virus exhibits a complex transcriptional pattern: one aspect of gene expression shared with the acquired immunodeficiency syndrome retrovirus. J. Virol. **61**:1325–1331.
8. **Emerman, M., R. Vazeux, and K. Peden.** 1989. The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. Cell **57**:1155–1165.
9. **Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46**:807–817.
10. **Felber, B. K., M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, and G. N. Pavlakis.** 1989. rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. Proc. Natl. Acad. Sci. USA **86**:1495–1499.
11. **Hammarskjold, M.-L., J. Heimer, B. Hammarskjold, I. Sangwan, L. Albert, and D. Rekosh.** 1989. Regulation of human immunodeficiency virus *env* expression by the *rev* gene product. J. Virol. **63**:1959–1966.
12. **Heaphy, S., J. T. Finch, M. J. Gait, J. Karn, and M. Singh.** 1991.

Human immunodeficiency virus type 1 regulator of virion expression, rev, forms nucleoprotein filaments after binding to a purine rich "bubble" located within the rev-responsive region of viral mRNAs. Proc. Natl. Acad. Sci. USA **88**:7366–7379.
13. **Huang, X., T. J. Hope, B. L. Bond, D. McDonald, K. Grahl, and T. G. Parslow.** 1991. Minimal Rev-response element for type 1 human immunodeficiency virus. J. Virol. **65**:2131–2134.
14. **Jaeger, J. A., D. H. Turner, and M. Zuker.** 1989. Improved predictions of secondary structures for RNA. Proc. Natl. Acad. Sci. USA **86**:7706–7710.
15. **Johnson, D. A., J. W. Gautsch, R. Sportsman, and J. H. Elder.** 1983. Improved technique utilizing non-fat dry milk for analysis of protein and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. **1**:3–7.
16. **Kim, S. Y., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63**:3708–3713.
17. **Kiyomasu, T., T. Miyazawa, T. Furuya, R. Shibata, H. Sakai, J.-I. Sakuragi, M. Fukasawa, N. Maki, A. Hasegawa, T. Mikami, and A. Adachi.** 1991. Identification of feline immunodeficiency virus *rev* gene activity. J. Virol. **65**:4539–4542.
18. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell. Biol. **108**:229–241.
19. **Le, S. Y., J. H. Chen, and J. V. Maizel.** 1989. Efficient searches for unusual folding regions in RNA sequences, p. 127–136. *In* R. H. Sarma and M. H. Sarma (ed.), Proceedings, 6th Conversation in Biomolecular Stereodynamics, vol. 1. Adenin Press, New York.
20. **Le, S. Y., M. H. Malim, B. R. Cullen, and J. V. Maizel.** 1990. A highly conserved RNA folding region coincident with the Rev response element of primate immunodeficiency viruses. Nucleic Acids Res. **18**:1613–1623.
21. **Liu, F. T., M. Zinnecker, T. Hamaoka, and D. H. Katz.** 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry **18**:690–693.
22. **Malim, M. H., S. Bohnlein, R. Fenrick, S. Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. Functional comparison of the Rev trans-activators encoded by different primate immunodeficiency virus species. Proc. Natl. Acad. Sci. USA **86**:8222–8226.
23. **Malim, M. H., S. Bohnlein, J. Hauber, and B. R. Cullen.** 1989. Functional dissection of the HIV-1 Rev trans-activator—derivation of a trans-dominant repressor of Rev function. Cell **58**:205–214.
24. **Malim, M. H., and B. R. Cullen.** 1991. HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency. Cell **65**:241–248.
25. **Malim, M. H., J. Hauber, S. Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) **338**:254–257.
26. **Mermer, B., B. K. Felber, M. Campbell, and G. N. Pavlakis.** 1990. Identification of trans-dominant HIV-1 rev protein mutants by direct transfer of bacterially produced proteins into human cells. Nucleic Acids Res. **18**:2037–2044.
27. **Michael, N. L., P. Morrow, J. Mosca, M. Vahey, D. S. Burke, and R. R. Redfield.** 1991. Induction of human immunodeficiency virus type 1 expression in chronically infected cells is associated primarily with a shift in RNA splicing patterns. J. Virol. **65**:1291–1303.
28. **Muesing, M. A., D. H. Smith, C. D. Cabradilla, C. V. Benton, L. A. Lasky, and D. J. Capon.** 1985. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature (London) **313**:450–458.
29. **Nicolaisen-Strouss, K., H. P. Kumar, T. Fitting, C. K. Grant, and J. H. Elder.** 1987. Natural feline leukemia virus variant escapes neutralization by a monoclonal antibody via an amino acid change outside the antibody-binding epitope. J. Virol. **61**:3410–3415.
30. **Nordeen, S. K., P. P. D. Green, and D. M. Fowlkes.** 1987. A rapid, sensitive, and inexpensive assay for chloramphenicol acetyltransferase. DNA **6**:173–178.
31. **Olsen, H. S., A. W. Cochrane, P. J. Dillon, C. M. Nalin, and C. A. Rosen.** 1990. Interaction of the human immunodeficiency virus type 1 Rev protein with a structured region in env mRNA is dependent on multimer formation mediated through a basic stretch of amino acids. Genes Dev. **4**:1357–1364.
32. **Pedersen, N. C., E. W. Ho, M. L. Brown, and J. K. Yamamoto.** 1987. Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. Science **235**:790–793.
33. **Phillips, T. R., R. L. Talbott, C. Lamont, S. Muir, K. Lovelace, and J. H. Elder.** 1990. Comparison of two host cell range variants of feline immunodeficiency virus. J. Virol. **64**:4605–4613.
34. **Rosen, C. A., and G. N. Pavlakis.** 1990. Tat and Rev: positive regulators of HIV gene expression. AIDS **4**:499–509.
35. **Saltarelli, M., G. Querat, D. A. Konings, R. Vigne, and J. E. Clements.** 1990. Nucleotide sequence and transcriptional analysis of molecular clones of CAEV which generate infectious virus. Virology **179**:347–364.
36. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
37. **Sarkar, G., and S. S. Sommer.** 1988. RNA amplification with transcript sequencing (RAWTS). Nucleic Acids Res. **16**:5197.
38. **Sodroski, J., W. C. Goh, C. Rosen, A. Dayton, E. Terwilliger, and W. Haseltine.** 1986. A second post-transcriptional trans-activator gene required for HTLV-III replication. Nature (London) **321**:412–417.
39. **Talbott, R. L., E. E. Sparger, K. M. Lovelace, W. M. Fitch, N. C. Pedersen, P. A. Luciw, and J. H. Elder.** 1989. Nucleotide sequence and genomic organization of feline immunodeficiency virus. Proc. Natl. Acad. Sci. USA **86**:5743–5747.
40. **Tiley, L. S., P. H. Brown, S. Y. Le, J. V. Maizel, J. E. Clements, and B. R. Cullen.** 1990. Visna virus encodes a post-transcriptional regulator of viral structural gene expression Proc. Natl. Acad. Sci. USA **87**:7497–7501.
41. **Tiley, L. S., M. H. Malim, and B. R. Cullen.** 1991. Conserved functional organization of the human immunodeficiency virus type 1 and visna virus Rev proteins. J. Virol. **65**:3877–3881.
42. **Vigne, R., V. Barban, G. Querat, V. Mazarin, I. Gourdou, and N. Sauze.** 1987. Transcription of visna virus during its lytic cycle: evidence for a sequential early and late gene expression. Virology **161**:218–227.
43. **Zuker, M.** 1989. On finding all suboptimal foldings of an RNA molecule. Science **244**:48–52.