Quantitative assays for Maedi-visna virus genetic sequences and mRNA’s based on RT-PCR with real-time FRET measurements

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Received 16 August 2002; accepted 14 October 2002

Abstract

We developed robust, ultrasensitive, and accurate quantitative assays for maedi-visna virus (MVV) RNA and DNA genomic sequences and mRNA’s expressed at various stages of lentiviral replication. Assay design was based on PCR with real-time fluorescence resonance energy transfer measurements. Specific assays were developed for gag-pol (genomic), tat, rev, env, and vif transcripts. Assay linearity ranged from 60 to 6 \times 10^7 copies of target DNA. All assays were able to detect and measure corresponding mRNA’s in MVV-infected FOS cells, whereas no signal was detected in mock-treated cells. In addition, RT-PCR based on amplification of gag sequences could be used to quantify RNA genomic sequences in supernatants from infected cells. These quantitative assays can be used to study the role of genetic elements in MVV infection and pathogenesis. They also allow rapid testing of lentiviral vectors and packaging systems based on MVV.

Keywords: Lentiviruses; Maedi-visna virus; LightCycler; mRNA-quantification; DNA quantification; RT-PCR; FRET; Splicing; mRNA

Introduction

The replication cycle of lentiviruses, a subfamily of retroviruses, consists of defined steps requiring several cis and trans genetic elements. Lentiviral genomes include three open reading frames (ORFs), gag, pol, and env, common to all retroviruses. In addition, they contain several accessory genes mostly implicated in regulation of viral gene expression. Maedi-visna virus (MVV) is a lentivirus that infects sheep resulting in chronic progressive pulmonary disease (maedi) and degenerate CNS affection (visna) (Sigurdsson et al., 1957; Petursson et al., 1991). MVV contains ORFs for transcriptional transactivator (tat), regulator of viral protein expression (rev), and vif of poorly characterized function.

Cells infected by MVV contain several different transcripts. A full-length genomic transcript (9.4 kb) also serves as mRNA for gag and pol. The major splice donor of MVV has the consensus sequence AAG/GTAAGA, and the splice junction is between nucleotides 304 and 305 of the viral RNA (Sonigo et al., 1985; GenBank Accession No. 470313). Three spliced transcripts of 5.0 kb for vif and 4.3 and 3.7 kb that contain the whole env ORF and two small multiply spliced transcripts of 1.8 and 1.5 kb for tat and rev are present in MVV-infected cells (Davis et al., 1987; Vigne et al., 1987). In the early phase of lentiviral infection, small multiplexed mRNA’s for regulatory proteins are expressed, followed by expression of larger mRNA’s (Vigne et al., 1987).

To facilitate characterization of function of MVV cis and trans genetic elements in vitro in cell culture and in vivo in the animal, we have developed robust, ultrasensitive, and accurate quantitative assays for specific DNA and RNA molecules present at various stages of viral replication. Assay design was based on PCR with real-time fluorescence resonance energy transfer (FRET) measurements. Assays were designed with two fluorochrome-labeled probes that hybridize adjacent to each other on target DNA (Fig. 1)

Fig. 1. Principle of fluorescence resonance energy transfer measurements. One hybridization probe is labeled with donor fluorochrome fluorescein (F). Excitation of the donor fluorochrome with light of correct wavelength results in FRET to Cy5 acceptor fluorochrome (Cy5) on adjacent probe. The emitted light signal from excited Cy5 correlates closely with amount of template available for probe binding because FRET is only effective over very short distances (<10 nm) between cohybridized probes. P, 3′ phosphate group, which prevents elongation from the acceptor probe during amplification.

(Wittwer et al., 1997a). One hybridization probe is labeled with a donor fluorophore, fluorescein. Excitation of the donor fluorochrome with light of correct wavelength causes FRET to the Cy5 acceptor fluorochrome on the adjacent probe. This results in measurable light emission of a longer wavelength, which correlates closely with amount of template available for probe binding. FRET is only effective over very short distances (<10 nm). Free probes in solution generate essentially no signal nor do probes spaced more than a few nucleotides apart on a template (Glazer and Mathies, 1997).

Assay specificity for the various mRNA’s expressed by MVV is achieved by using carefully selected primer-binding sites and probes resulting in FRET between donor and recipient fluorescent probes straddled across splice sites. The combination of PCR amplification coupled with real-time FRET measurement creates ultrasensitive assays capable of detecting less than zeptomolar (<10⁻¹⁰ M) amounts of nucleic acid sequences.

Results

Several primers were designed to create (RT)-PCR-FRET assays for MVV genomic RNA, proviral DNA, and various mRNA’s. Assay specificity was achieved by using two strategies: (1) binding sites for PCR primers were carefully selected so that amplification would only occur if message was spliced, because unspliced RNA would be too long for efficient amplification. In addition, binding sites for certain primers were only retained in some mRNA’s but not others. (2) Binding sites for FRET probes were on each side of spliced introns. With this arrangement, FRET would only occur in correctly spliced transcripts. Based on this strategy we were able to select appropriate sets of amplification primers and FRET probes to design assays to measure specific transcripts (Table 1 and Fig. 2).

An assay for quantitative analysis of MVV DNA was developed using plasmid p8XSp5 as standard. This plasmid includes the 5′ sequence of clone KV1772-kv72/67, as previously described (Skraban et al., 1999). Amplification primers and FRET probes for gag sequences were designed, to amplify genomic RNA but not mRNA’s spliced by the major 5′ splice donor (Table 1). PCR-FRET resulted in a linear range when between 60 and 60 million copies were

Table 1
Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5′–3′</th>
<th>mRNA</th>
<th>Genomic localizationa</th>
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<tbody>
<tr>
<td>A. Primers</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gag-877F</td>
<td>AGC AAC TCT ACC CCA ACT TA</td>
<td>gag/pol</td>
<td>877–896</td>
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<tr>
<td>Gag-1092R</td>
<td>GCT CTA TTC CCA GCC ATC AT</td>
<td>gag/pol</td>
<td>1111–1092</td>
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<tr>
<td>Vif-235F</td>
<td>TGA GGA CCG GCA GAG TAA C</td>
<td>vif</td>
<td>235–256</td>
</tr>
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<td>Vif-431R</td>
<td>TCT TTT GGT GCC GGT ATG AAC TTA</td>
<td>vif</td>
<td>4992–4969</td>
</tr>
<tr>
<td>v-208F</td>
<td>GTT ACG GGA CCA GGG ACG CTC TCC</td>
<td>rev, env</td>
<td>208–231</td>
</tr>
<tr>
<td>Env-441R</td>
<td>CCA GTC CCT GCT GTT CTT TCC</td>
<td>env</td>
<td>6091–609</td>
</tr>
<tr>
<td>Env-2456R</td>
<td>TGT TAG TCT TTG GCC AGG T</td>
<td>tat, env</td>
<td>5779–5761</td>
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<tr>
<td>Env-2856R</td>
<td>AAC CTC GGC TAA ACA CTT TTC AC</td>
<td>env</td>
<td>6201–6179</td>
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<tr>
<td>Rev-484R</td>
<td>GGC TCC TAT CAC CAG TGA TCG</td>
<td>rev</td>
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<td>Tat-406F</td>
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<tr>
<td>Tat-824R</td>
<td>TCC ACC GGT GCT TCT ATC A</td>
<td>tat</td>
<td>8594–8576</td>
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<tr>
<td>B. Probes</td>
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<tr>
<td>Gag-Flu</td>
<td>CAA ACA GTG GCA ATG CAG CAT GG-P</td>
<td>gag/pol</td>
<td>987–1009</td>
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<tr>
<td>Gag-Cy5</td>
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<td>gag/pol</td>
<td>1011–1033</td>
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<td>SA1</td>
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<td>rev, env, vif</td>
<td>4854–4876</td>
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<tr>
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<td>TTT TCT TGG CCA TCC TAG ACC-P</td>
<td>rev, env</td>
<td>5950–5976</td>
</tr>
<tr>
<td>SA3</td>
<td>GAT GGA TTA CTC TTA CCA GCA TT-F</td>
<td>tat, env</td>
<td>5626–5648</td>
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<tr>
<td>Major-SD</td>
<td>C5-CTT ACT TCA GGC GTC CCC GAA GC-P</td>
<td>rev, env, vif</td>
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<td>6073–6095</td>
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<tr>
<td>Tat-779-Cy5</td>
<td>C5-CTT ACA ACA ACG TAA AGA AGA TC-F</td>
<td>tat</td>
<td>8531–8553</td>
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a Genomic localization in molecular clone KV1772-kv72/67 (Andresson et al., 1993; GenBank Accession No. 265825).
b Abbreviations of modifications of molecular probes: Cy5, Cy5 dye; F, fluorochrome; P, 3′ phosphate.
included per reaction (6 orders of magnitude). The results from these were used for construction of a standard curve (Fig. 3). Intraassay coefficient of variation (CV) for the assay was 12% and interassay CV was 17%. Efficiency of amplification was 1.88×/cycle. To demonstrate the use of this assay for quantification of RNA genomic sequences, RT-PCR based on amplification of gag sequences was carried out in supernatants from infected cells. We measured...
3 × 10^4 molecules/µl MVV genomic RNA in supernatant, whereas no signal was generated in mock-transfected control cells.

For the quantification of the vif transcript, primers Vif-235F and Vif-431R were used (Fig. 2). The forward primer was situated 5' of the major splice donor. The reverse primer was located 3' of start codon of the vif ORF and in a region spliced out of other MVV mRNA’s. Amplification from infected fetal ovine synovial (FOS) cells resulted in a product of 219 bp (Fig. 4A), which was cloned in PCR-Script plasmid and sequenced. The sequencing revealed that the splice acceptor site at 4843 was used for the vif tran- script, confirming previously described results (Vigne et al., 1987). The assay could quantify 60 to 60 million copies of target DNA (Fig. 5). The intra-assay CV for the vif assay was 13% and inter-assay CV was 15%. Efficiency of amplification was 1.91×/cycle. When electrophoresis of PCR products on agarose gels (Fig. 4A) is compared to results from the real-time FRET measurements (Fig. 5), the advantage of using the latter method for quantification is shown. A difference in quantity between the highest standards (60,000 to 60 million molecules/µl) could not be detected on agarose gel, and a product in the reaction using the

![Fig. 3. PCR-FRET amplification of gag-pol sequences from genomic DNA template. The template was linearized plasmid p8XSp5, which contained the 5' sequence of clone KV1772-kv72/67 including relevant gag-pol region. For gag-pol primers and probes, see Table 1 and Fig. 2. (A) Amplification curves based on real-time fluorescence measurements during PCR. Assays were performed in duplicate on seven samples representing a 10-fold dilution series ranging from 60 to 60 million copies of template. (B) Semilogarithmic transformation of amplification curves in A. (C) Standard curve demonstrating a linear relationship between logarithm of copy number of template versus PCR cycle number. PCR cycle number was calculated from intersections of extrapolated straight lines between two lowest points in each curve in B and background noise band. The standard curve was linear over 6 orders of magnitude.](image)

![Fig. 4. PCR products created by reverse transcription and amplification of MVV mRNA’s from KV1772-kv72/67-infected FOS cells. For primers and probes, see Table 1 and Fig. 2. Products (2 µl) after 45 cycles of PCR were electrophoresed in 1.7% agarose. Size marker was X174 DNA cut with HaeIII. (A) Amplification with primers Vif-235F and Vif-431R for the vif product (second lane from the right). Vif cDNA was cloned in plasmid and used in a linearized form in lanes 2–9 to generate a standard curve. End point analysis is very inferior to real-time PCR-FRET curves in Fig. 5. (B) Amplification with primers v-208F and Env-441R for the small env product. (C) Amplification with primers v-208F and Env2-456R for the large env product. (D) Amplification with primers v-208F and Env2-859R for the large env product. (E) Amplification with primers Tat-406F and Tat-824R for the tat product. (F) Amplification with primers v-208F and Rev-484R for the rev product.](image)
standard did not rise above background. Curves from mock-treated cells and samples with 6 copies of amplified light brown curve (marked with an asterisk in A and B) represents cDNA amplicons shown in Fig. 4A. For explanation of graphs in A–C, see the legend to Fig. 3. The light brown curve (marked with an asterisk in A and B) represents cDNA amplification from FOS cells infected with KV1772-kv72/67. Other curves represent amplifications from 60 to 60 million copies of cloned template as standard. Curves from mock-treated cells and samples with 6 copies of standard did not rise above background.

lowest standard (60 molecules/μl) could not be detected (Fig. 4A).

For an assay to quantify the small (3.6 kb) env transcript, primers v-208F and Env-441R were used to form PCR products. The forward primer was positioned 5’ of the major splice donor site at 304. The reverse primer was located 3’ of splice donor site at 6097 in a region absent from the tat and rev transcripts (Fig. 2). PCR using these primers resulted in amplification of two amplicons of 289 and 243 bp (Fig. 4B). Both products were cloned into PCR-Script plasmid and sequenced. The two products resulted from differential splicing. The smaller fragment, cloned in construct pENV-2ex, was a product of one splice event, where splice acceptor at site 5946 was used. The larger fragment, cloned in construct pENV-3ex, was the product of two splice events and included a small fragment of the pol gene (4843–4888). This fragment is also included in tat and rev transcripts (Gourdou et al., 1989; Mazarin et al., 1988). The second splice acceptor was at site 5946 as in the single-spliced message (Fig. 2). Specific assays for both splice variants were developed. Probe Major-SD was used with probe SA1 for the three exon transcript and with probe SA2 for the two exon transcript (Fig. 2). Standard curves were made and both assays allowed quantification of 60 to 60 million copies of target DNA. Intraassay CVs for the assays for pENV-3ex and pENV-2ex were 6 and 14%, respectively. Interassay CVs were 12% for pENV-3ex and 17% for pENV-2ex. Efficiency of amplification for the pENV-3ex assay was 1.92×/cycle, and for the pENV-2ex assay, 1.97×/cycle.

The cloning of a PCR product representative of the larger (3.9 kb) env mRNA was carried out using primer v-208F as forward primer coupled with either reverse primer Env2-456R or reverse primer Env2-856R (Table 1 and Fig. 2). Using reverse primer Env2-456R with v-208F also resulted in amplification of the multiply spliced tat mRNA, which utilizes the splice site at 5625 (Fig. 2). PCR using these primers created products of 251 and 297 bp (Fig. 4C), which were cloned in pBluescript to construct plasmids pET-2ex and pET-3ex, respectively. Sequencing of the plasmid inserts revealed that their size difference resulted from differential splicing of the small fragment from the pol gene discussed above. The second splice event occurred with site 5625 as splice acceptor (Fig. 2). Probes for fluorescence detection were selected across the different splice junction sites. Probes Major-SD and SA3 were used for the two exon transcript (pET-2ex), and probes Major-SD and SA1, for the three exon transcript (pET-3ex) (Fig. 2). Standard curves were made and both assays were linear over the range of 60 to 60 million copies of target DNA. Intraassay and interassay CVs for pET-3ex were 16 and 12%, respectively. Intraassay and interassay CVs for pET-2ex were 9 and 14%, respectively. Efficiency of amplification for the two assays was 1.98×/cycle for pET-3ex and 1.96×/cycle for pET-2ex. With the pET-3ex and pET-2ex assays we were unable to distinguish between the tat transcript and the large env transcript.

Using forward primer v-208F coupled with reverse primer Env2-856R (Fig. 2) resulted in amplification of several PCR products in various amounts (Fig. 4D). Primer Env2-856R has the advantage of being complementary to a sequence absent from tat and rev transcripts. The two smaller bands (352 and 397 bp) were the results of amplification of the smaller env mRNA, but two larger fragments were from the amplification of the larger env transcript, resulting in PCR products of 673 and 718 bp. The larger PCR products were cloned into pBluescript, to construct pENVL-2ex and pENVL-3ex, respectively. The fragments were sequenced, the size difference being the result of splicing of the pol fragment into the larger splice variant. Quantitative assays for these transcripts were developed using fluorescent probes Major-SD and SA3, for the two-exon transcript, and Major-SD and SA1, for the three-exon transcript. Both assays were linear over the range of 600 to 60 million copies of target DNA. The intraassay CV for
pENVL-3ex was 16%, the interassay CV was 14%, and efficiency of amplification for the assay was 1.69×/cycle. The intraassay CV for pENVL-2ex was 20%, the interassay CV was 13%, and efficiency of amplification was 1.73×/cycle. Even though amplification with primers v-208F and Env2-856R generated both large and small env transcripts, the assay was made specific for large env mRNA’s by using probes Major-SD and SA3 (Fig. 2). There would, however, be fluorescence detection of the small env mRNA from cells if the SA1 probe were used.

The PCR product using primers Tat-406F and Tat-824R representative for the tat mRNA (Table 1 and Fig. 2) resulted in amplification of 434-bp cDNA from KV1772-kv72/67 infected FOS cells (Fig. 4E). The forward primer was located 5’ of SA 5946, used in generating rev transcripts. This design distinguishes tat mRNA from the mRNA of rev (Fig. 2). The reverse primer was located 3’ of SA 8530, making formation of large PCR products from genomic RNA, or vif and env mRNA’s, improbable because of their length. The 434-bp fragment was cloned into TA-cloning vector. Sequencing of a plasmid clone (pTAT) showed the sequence of the tat cDNA to be identical to the previously described sequence of tat mRNA (Gourdon et al., 1989). Probes for fluorescence detection, Tat-753-Flu and Tat-779-Cy5, were selected across the third splice junction of the transcript. This design resulted in an assay specific for the tat transcript. The tat assay was linear over the range of 60 to 60 million copies. Intraassay CV was 14% and interassay CV was 18%. Efficiency of amplification for the tat assay was 1.96×/cycle.

For the cloning of a PCR product for the rev transcript, primers v-208F and Rev-484R were used. The forward primer was positioned 5’ of the major splice donor and the reverse primer was 3’ to the splice acceptor site 8530 (Fig. 2). Amplification with these primers on MVV cDNA resulted in three amplicons, of 350, 305, and 154 bp (Fig. 4F). A possible tat transcript amplification of 672 bp from FOS cytoplasm was not detected. All three PCR products were cloned and the inserts were sequenced. The smallest fragment represented two exons (pREV-2ex) resulting from a splicing event between the major splice donor 304 and splice acceptor 8530. The middle fragment contained three exons (pREV-3ex) with the first splice acceptor 5946 and the latter splice event between splice donor 6097 and splice acceptor 8530. The large fragment (pREV-4ex) had four exons, created by three splicing events, as previously described (Mazarin et al., 1988). An assay for the multiply spliced transcript was developed for pREV-4ex and pREV-3ex. Probes Major-SD and SA1 were used for the four-exon transcript, and probes Major-SD and SA2, for the three-exon transcript (Fig. 2). A linear range from 60 to 60 million copies of target DNA was noted with both assays. Intraassay CV was 10% for the pREV-3ex assay and 18% for the pREV-4ex assay and the interassay CVs were 9 and 18%, respectively. Efficiency of amplification was 1.87×/cycle for both assays.

We determined if the assays could measure level of transcripts in cell culture. FOS cells were transfected with cloned replication-competent MVV in plasmid LV1-1KS2 or infected with molecular clone KV1772-kv72/67. Cells were harvested and cytoplasm was collected at various stages posttransfection or postinfection. Assays for all different MVV RNAs measured the specific transcripts in various amounts in all tested samples. Levels of all transcripts were within the linear range of the corresponding assay and considerably higher than the lowest standards. Levels were generally from 10^2 to 1.5 × 10^7 molecules/μl for different mRNA’s. In contrast, no signals for any of the assays were generated in mock-transfected controls, demonstrating their specificity.

**Discussion**

We have designed primers and probes for quantitative assays to measure MVV RNA genome and DNA proviruses as well as transcripts for gag-pol, vif, tat, env, and rev. We defined PCR conditions for efficient amplification and quantification of these nucleic acid sequences using real-time fluorescence measurement. Design of assays allows specific measurements of individual transcripts. PCR-FRET resulted in high sensitivity with the capability of quantifying nucleic acid templates to as few as 60 copies per reaction. Only the assay for the larger env mRNA had higher detection limits, 600 copies per reaction, presumably because of the length of the amplicon. This amount is much lower (often orders of magnitude) than levels of transcripts present in most infected cell culture preparations. Assay sensitivity facilitates measurement of MVV genetic elements in biologic material available in very limited quantity or where MVV is present at low levels. Because assays were linear over 6 orders of magnitude, analytic levels should very rarely be outside of the measurement range. The assays are robust, reagents are relatively cheap, and labor is modest. The assays do, however, rely on a highly specialized equipment, the LightCycler instrument. In this thermocycler, PCR analysis is carried out in capillary glass reaction cuvettes allowing rapid heat exchange. This results in small intrasample temperature variation despite fast (i.e., 30 min) 45-cycle PCR assays. This is optimal design for analytical work based on real-time fluorescence detection. There is no reason, however, why these assays could not be modified for use in other PCR instruments with real-time fluorescence capability. The same primers and probes could be used because the fluorochromes used require excitation and emission wavelengths commonly available on such instruments. Conditions for PCR would, however, need to be optimized for each type of instrument. If genetic variation coincides with primer- or probe-binding sites, adjustment in their sequences should...
be straightforward. Similar quantitative assays could also be developed for other lentiviruses.

The MVV mRNA’s have very similar 5’ and 3’ ends, and the problem of contamination of an assay by amplification of other mRNA’s was a concern, for example, would-be formation of a 672-bp Tat transcript when using primers for the rev assays. However, this transcript apparently could not compete with shorter transcripts in RT-PCR analysis, perhaps in part because of the short duration of the elongation step in PCR analysis (12 s).

The quantitative assays described in this work can be used to study the role of genetic elements in MVV infection and pathogenesis. They also allow rapid testing of lentiviral vectors and packaging systems based on MVV. The assays could also be further developed and investigated as diagnostic tests for MVV infection, including assessing virus load and stage of infection.

Materials and methods

Primers and probes

PCR primers and FRET hybridization probes for MVV RNA of molecular clone KV1772-kv72/67 (Andresson et al., 1993; GenBank Accession No. 265825) were designed by using Oligo (National Biosciences, Inc., Plymouth, MN) and MacVector (Oxford Molecular Group, Oxford, UK) computer programs. Sequences of PCR primers and hybridization probes are shown in Table 1. A schematic localization of oligonucleotides in the MVV genome and mRNA’s is shown in Fig. 2.

Infection and transfection of FOS cells

FOS cells were grown at 37°C in humidified atmosphere of 5% CO₂ in Dulbecco’s minimal essential medium (DMEM) (GibcoBRL Life Technologies, Paisley, UK) supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, and either 10% lamb serum (growth medium) or 1% lamb serum (maintenance medium). Subconfluent monolayers of FOS cells were infected with supernatant from sheep macrophages infected with molecular clone KV1772-kv72/67 (Andresson et al., 1993) using standard techniques (Ausubel et al., 1994). Cells were harvested for mRNA isolation 48, 72, and 96 h postinfection. Viral transcripts from infected FOS cells were used for cloning of PCR products by RT-PCR.

To generate mRNA for quantification, 5 µg of molecular clone LV1-1KS2 (Staskus et al., 1991) was transfected in FOS cells using calcium phosphate coprecipitation method (Graham and van der Eb, 1973). Transfected cells were incubated in DMEM with 10% newborn calf serum for 48 h. In all experiments mock-treated cells (transfected without plasmid) were used as negative controls.

RNA isolation

Total RNA was isolated from cytoplasm of infected FOS cells using RNA isolation kit (Stratagene, La Jolla, CA). The manufacturer’s instructions were scaled down 20 times. Viral particles from supernatant culture fluid were concentrated with ultracentrifugation in Beckman L5-50E at 50,000 g for 90 min and resuspended in 140 µl phosphate-buffered saline. Viral RNA was isolated from supernatant using QIAamp viral RNA mini kit (QIAgen, Venlo, the Netherlands) according to the manufacturer’s instructions.

To eliminate DNA contamination, RNA samples were treated with 1 µl DNase (2 U/µl; Ambion, Austin, TX) in 10× transcription buffer (Ambion) for 20 min at 37°C and 15 min at 70°C.

Reverse transcriptase reaction

cDNA was constructed using the Thermoscript RT-PCR system (Life Technologies) according to the manufacturer’s instructions. Random hexamers (50 ng/µl) were used to prime reverse transcriptase. The reaction samples were incubated at 25°C for 10 min, 50°C for 50 min, and 85°C for 5 min.

PCR-FRET

PCR was carried out in a LightCycler (Idaho Technology, Idaho Falls, ID) (Wittwer et al., 1997b) by using linearized plasmids as templates. Reaction samples (10 µl) contained 200 µM each dNTP, 0.5 µM primers, 0.2 µM fluorescent probes, 0.08 U/µl KlenTaq1 polymerase (Ab Peptides, Inc., St. Louis, MO) diluted in enzyme diluent buffer (10 mM Tris, pH 8.3, 250 µg/ml BSA; Idaho Technology), and reaction buffer (50 mM Tris, pH 8.3, 4 mM MgCl₂, 250 µg/ml BSA; Idaho Technology). Mixing and dilutions were carried out in Eppendorf tubes lubricated with silicon. All assays were performed in duplicate. PCR products were characterized by electrophoresis on 1.7% agarose gels, using φX174 (250 ng) cut with HaeIII as size marker.

Temperature profiles of PCR assays consisted of initial denaturation step for 2 min at 94°C, followed by 45 cycles of denaturation for 0 s at 94°C, annealing with fluorescence monitoring for 0 s at 58°C, and elongation at 72°C. Elongation time was set depending on PCR product length: 10 s for the gag, vif, pET-2ex, pENV-2ex, and pENV-3ex assays; 12 s for the tat, pREV-3ex, pREV-4ex, and pET-3ex assays; and 22 s for the pENVL-2ex and pENVL-3ex assays.

Calculations of amplification efficiency and coefficients of variation

Efficiency of amplification was calculated based on differences in the number of cycles (n and m) when amplified
DNA in two PCR assays with different amounts of original template (D is the ratio of difference) reached same baseline level above limit of detection. With these parameters, efficiency of amplification $X$ (max. 2) was calculated as the common ratio from geometric series using the following formula: $X = 10^{\log D - m}$. CVs were calculated based on differences between duplicate assays. Mean CVs were based on analysis of at least seven duplicates.

**Cloning and manipulation**

PCR products were cloned with TA-cloning vector pCR2.1 (Invitrogen, Groningen, The Netherlands) for tat and the three-exon rev PCR products. Other PCR products were cloned using PCR-Script Amp cloning kit (Stratagene). Both kits were used according to the manufacturers’ instructions. The PCR product representing the larger (4.3 kb) mRNA generated with primers containing XbaI heels (Table 1) was cloned in XbaI-digested pBluescript. Plasmids were transformed and grown in Escherichia coli strain DH5α F’IQ (Life Technologies).

Inserts in plasmid clones (400 ng) were sequenced with ABI Prism BigDye terminator cycle sequencing ready reagent kit (PE Biosystems, Foster City, CA) on ABI Prism 377 DNA sequencer. Sequencing was carried out using primers T7, either primer T3 for inserts in PCR-Script Amp cloning, or primer Met for inserts in TA-cloning vector.

Plasmids were digested with a restriction enzyme cutting at a single site outside of intended PCR region. Following linearization of plasmids and purification with GFX PCR DNA and gel band purification kit (Amersham Biosciences Inc., Piscataway, NJ) the DNA concentration was determined with Hoefer DyNA Quant 200 fluorometer (Amersham Biosciences Inc., Piscataway, NJ), and the corresponding copy number was calculated. Serial dilutions from 60 million to 60 copies of plasmids per microliter were done corresponding copy number was calculated. Serial dilutions from 60 million to 60 copies of plasmids per microliter were done in TLE (10 mM Tris, pH 8.0, 0.1 M EDTA) in tubes lubricated with silicon.

**Acknowledgments**

This work was supported by grants from The Icelandic Research Council Science Fund and Landspitali Science Fund. We thank Dr. Olafur S. Andresson and Dr. Valgerdur Andresdottir for the p8XSp5 plasmid and FOS cells and Dr. Katherine Staskus and Dr. Ashley Haase for the LV1-1KS2 plasmid. We thank Gudmundur H. Gunnarsson for expert assistance in preparation of the figures.

**References**


