

The extended packaging sequence of MoMLV contains a constitutive mRNA nuclear export function

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Abstract The present report shows that incorporation of defined sequences from the Moloney murine leukaemia virus (MoMLV) into Rex dependent expression vectors based on the human T-cell leukaemia virus (HTLV-1) allows Rex independent gene expression. Deletion mutagenesis of the MoMLV derived sequences allowed this function to be localised to a 312 nt length sequence overlapping the MoMLV gag p15/p12 open reading frame. This 'extended packaging sequence' has been reported to markedly increase the titre of in vitro packaged retroviral vectors. Using fluorescent in situ hybridisation combined with confocal microscopy we show that the 312 nt element can replace Rex mediated nuclear export and expression of transcripts containing HTLV-1 *cis* acting repressive elements. Our observations are consistent with the extended packaging sequence of MoMLV exerting a constitutive mRNA nuclear export function.

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Key words: mRNA nuclear export; Moloney murine leukemia virus; Extended packaging sequence; Human T-cell leukemia virus-1; Rex

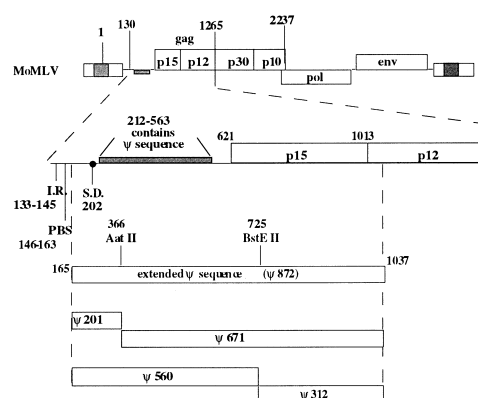
1. Introduction

Expression of genomic and subgenomic transcripts in complex retroviruses is regulated in part at the level of RNA export from the nucleus. *Cis* acting repressing sequences (CRS) in human immunodeficiency virus (HIV) [1–3], human T-cell leukaemia virus (HTLV)-2 [4,5], and HTLV-1 [5,6] act to retain the corresponding transcripts in the nucleus of the infected cell. Export of these transcripts is achieved through the action of viral gene products (HIV Rev and HTLV Rex) that interact with *cis* acting viral sequences (HIV RRE and HTLV RxRE) and actively transport the corresponding transcripts [7–10].

Simian retrovirus type 1 (SRV-1), SRV-2, and Mason-Pfizer monkey virus (MPSV), three members of the family of the type D simian retroviruses, have been shown to contain *cis* acting RNA transport elements (CTE) that can overcome the Rev regulation of HIV-1 [11–14]. Similar *cis* acting elements were reported in the 3' untranslated region of Rous sarcoma virus (RSV) RNA [15], in murine intracisternal-A particles [16], as well as in DNA viruses [17,18].

The packaging function of various Moloney murine leukaemia virus (MoMLV) sequences is localised to a sequence im-

A



B

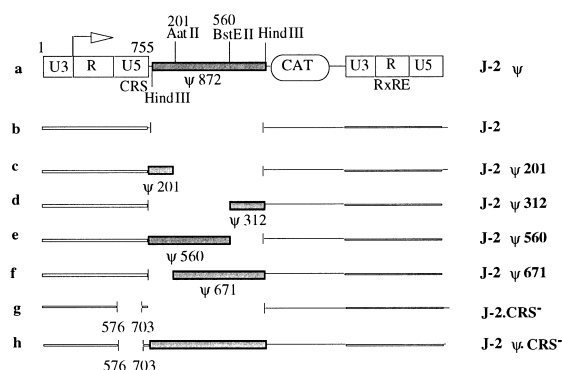


Fig. 1. Schematic representation of the MoMLV sequences and HTLV-1 LTR based expression constructs used in this study. The read out system was based on Tax dependent expression of the CAT gene from HTLV-1 LTRs. A: Sequences corresponding to the extended packaging element of MoMLV were derived from pHMB, an MoMLV based retroviral vector. The complete ψ^+ consisted of the canonical packaging sequence, nucleotides 165–725, and the extended sequence, nucleotides 725–1037. The latter overlapped with the entire gag p15 and the beginning of gag p12 open reading frame. B: The complete ψ^+ sequence or fragment derived from it were cloned in expression vectors based on HTLV-1 LTRs. The fragments were inserted in an appropriate *Hind*III site just downstream of the 5' LTR. The constructs contained two complete LTRs and a full ψ^+ sequence (a), or lacked the ψ^+ sequence (b), or lacked both the ψ^+ and the CRS element of the 5' LTR (c), or contained the ψ^+ but lacked the CRS (d), or had truncations of the ψ^+ sequence as depicted (e–h). Construct f contains the MoMLV gag derived sequences, while construct g contains the canonical MoMLV packaging sequence excluding the so called extended sequence from the gag gene.

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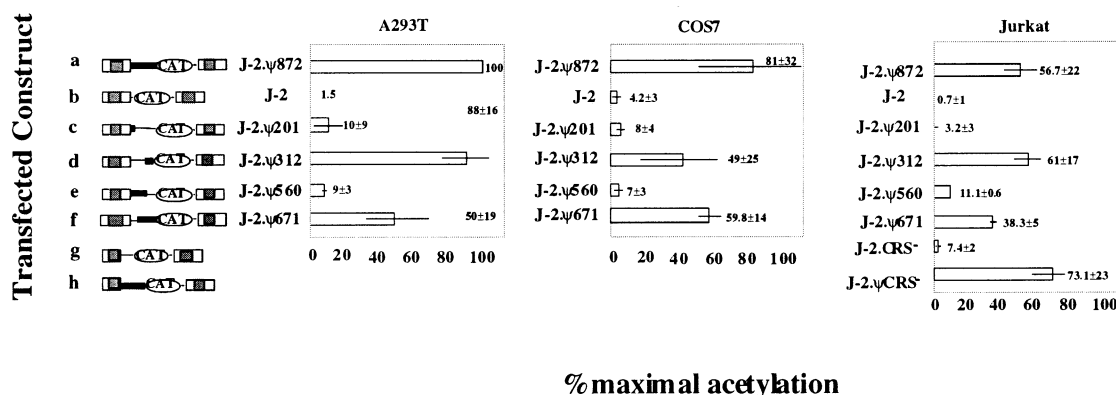


Fig. 2. Reporter CAT assay showing the result of cloning of ψ^+ sequences in HTLV-1 derived expression vectors, and the additional effect of the removal of the HTLV-1 5' CRS. Jurkat T cells were transfected using the DEAE-dextran method with 3 μ g of each HTLV-1 based plasmid and 1 μ g of Tax expressing plasmid and/or 0.1 μ g of Rex expressing plasmid. Cells were harvested 48 h later for CAT assays. COS7 and A293T cells were calcium phosphate transfected with 10 μ g of each CAT expressing plasmid with 2 μ g and 0.2 μ g of Tax and Rex expressing plasmids, respectively. The results shown for COS7 cells are from three, A293T cells from two, Jurkat cells from six experiments. CAT assays were kept in the linear range of acetylations. Bars represent experimental variations calculated as standard deviations. The % of maximum expression refers to the CAT signal in the presence of Tax as compared to the presence of Tax and Rex, in each individual case.

mediately neighbouring the 5' LTR designated as psi. Sequences further downstream overlapping the gag open reading frame were reported to markedly increase the titre of in vitro packaged retroviral vectors as compared to vectors containing only the psi sequence, leading to the assumption that the retrovirus packaging signal extends into the gag region [19–21]. In the present report we have investigated the influence of this sequence on the nuclear/cytoplasmic transport and expression of HTLV-1 LTR driven transcripts that contain CRS elements.

2. Materials and methods and results

A series of constructs were made by combining HTLV-1 LTR based expression vectors [6] with the complete extended ψ^+ or fragments thereof (Fig. 1A). The extended packaging signal of MoMLV, as corresponding to sequences spanning nucleotides 165–1037 of the viral RNA (Fig. 1A), was derived from the pHMB retroviral vector [19,20], using the following PCR primers: TAG TAC ACA GTT GGG GGC TCG TCG GG and AGC TGG AAG ATC TTC TAC CAT CGA TGG CCC A. The amplified fragment was confirmed by DNA sequencing. The splice donor and Gag start codons including the glycosylated Gag start codon (CTG) were mutated in

pHMB to be non-functional. These mutations ensured that spurious splicing or premature initiation of translation would not influence the observed mRNA transport or CAT activities.

J-2 ψ contained two complete HTLV-1 LTRs, the extended packaging sequence (ψ^+) of MoMLV nucleotides 165–1037, and the chloramphenicol acetyltransferase gene (CAT) [22] cloned downstream of the ψ^+ in place of the viral genes (Fig. 1B, a). The control vector J-2 did not have any MoMLV packaging sequences (Fig. 1B, b). J-2 ψ 201, J-2 ψ 312, J-2 ψ 560, J-2 ψ 671 were derived from the complete HTLV-1 LTRs and the corresponding fragments of the MoMLV ψ^+ (Fig. 1B, c–f). J-2.CRS $^-$ was similar to the control vector J-2 except that it contained an internal deletion in the 5' LTR of HTLV-1 nucleotides 576–703 (Fig. 1B, g), reported to be an RNA CRS element [5]. J-2 ψ .CRS $^-$ lacked the CRS element but contained a complete ψ^+ (Fig. 1B, h). HTLV-1 Tax and Rex were expressed from the pRK-7 plasmid (Dr David Goeddel, unpublished data) [23] with CMV immediate early promoters.

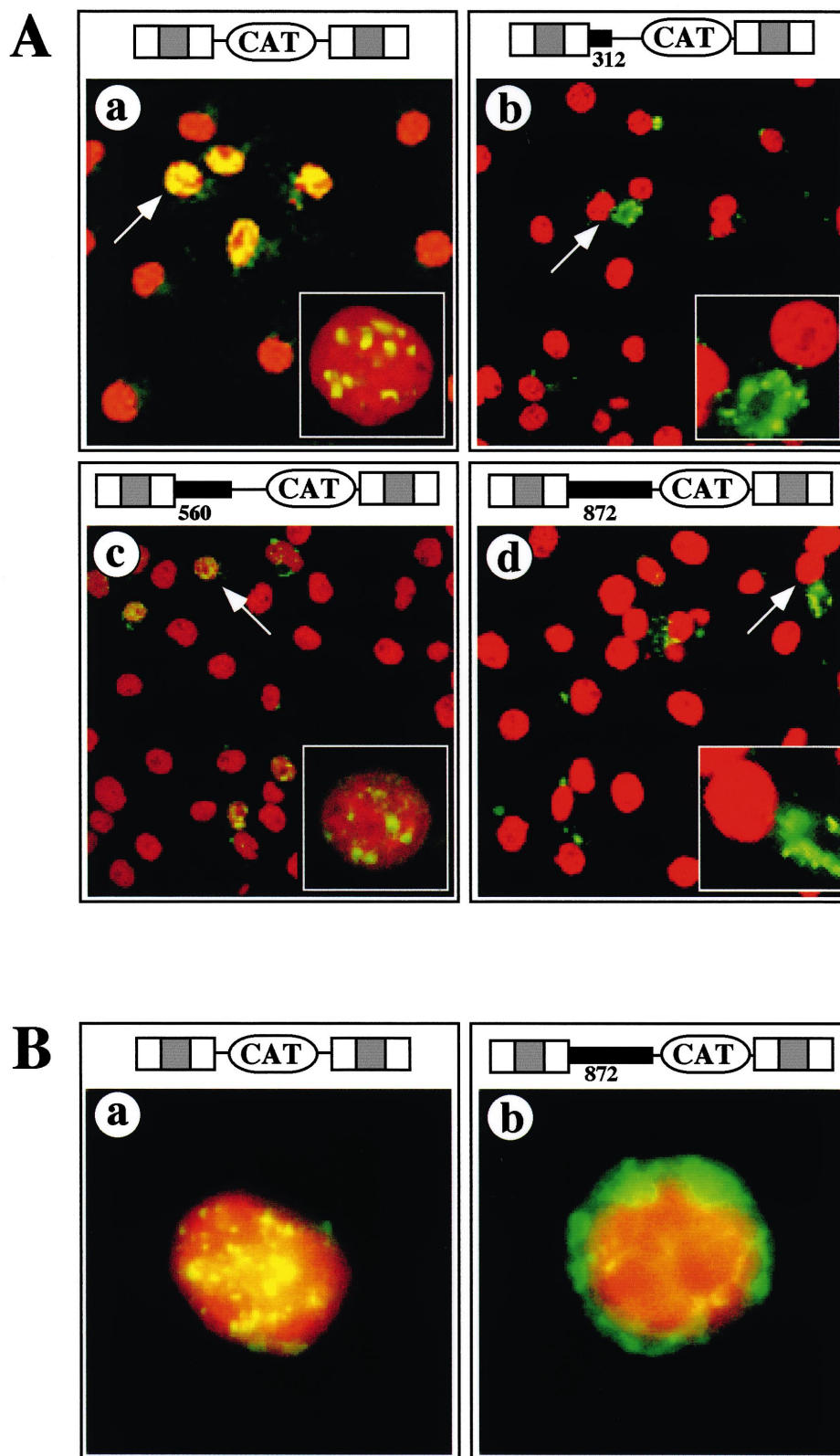
The constructs were based on HTLV-1 LTRs and therefore, in order to express the CAT gene, needed both Tax (transcriptional transactivation of the LTR promoter) and Rex (nuclear export of transcripts encoding CRS elements contained in

Fig. 3. Analysis of the effect of sequences derived from MoMLV ψ^+ on the subcellular localisation of the CAT encoding mRNAs using FISH and confocal microscopy, in COS7 cells (A) or A293T cells (B). Calcium phosphate transfections of 10 μ g of each CAT expressing plasmid and 2 μ g of Tax expressing plasmids were performed 48 h prior to FISH analysis. Higher magnifications are shown in the inserts. A: Subcellular localisation of transcripts driven by the control J-2 vector containing two complete HTLV-1 LTRs, in the presence of Tax indicates nuclear entrapment of transcripts appearing as punctuate intranuclear dots (a). Insertion of 312 bp sequence derived from the 5' region of MoMLV gag gene promoted export of CAT transcripts, visualised by green cytoplasmic staining (b). The canonical 560 bp packaging sequence of MoMLV failed to promote mRNA export (c), while the ψ^+ sequence that included both fragments induced efficient nuclear export of the CAT transcripts (d). B: The J-2 control vector transfected with Tax into A293T cells expressed CAT transcripts that could not leave the nucleus (a), while insertion of the ψ^+ into this vector promoted nuclear export of the transcripts (b). The single A293T cell is meant to show the difference in appearance of the two counterstaining procedures, using propidium iodide vs DAPI. The J-2 plasmid was used as template in a nick translation labelling reaction which incorporated digoxigenin-11-dUTP in place of dTTP. Probe was visualised using 10 μ g/ml fluorescein conjugated sheep anti-DIG antibody (Boehringer Mannheim). In A propidium iodide was used as a nuclear counter stain to stain total nucleic acids (red). A fluorescein-tagged anti-DIG antibody was used to detect the labelled probe (green). The red and green images were acquired simultaneously. Where the two colours overlap a yellow coloration was observed, indicating a nuclear localisation of the transcripts. Images were acquired using a Zeiss (LSM 410) confocal laser scanning microscope fitted with an argon ion laser and a helium neon laser using an oil immersion 63 $\times$ plan-APOCHROMAT lens (Zeiss). Boxed figures are enlargements of marked cells (white arrows). In B, cells were counterstained with DAPI. Red and green images were simultaneously obtained by excitation of propidium iodide and fluorescein at 488 nm using a Photometric cooled charge coupled device (CCD) camera. Images for DAPI and FITC were overlaid in Adobe Photoshop.

HTLV-1 LTRs). Jurkat T cells (10 million) were transfected via the DEAE-dextran method with 3 μ g of each HTLV-1 based plasmid and 1 μ g of Tax expressing plasmid and 0.1 μ g of Rex expressing plasmid, or with Tax alone. COS7, HeLa and A293T cells were calcium phosphate transfected

with 10 μ g of each CAT expressing plasmid with 2 μ g and 0.2 μ g of Tax and Rex expressing plasmids, respectively. Cells were harvested 48 h later for CAT assays.

J-2. ψ 872 produced high levels of Rex independent CAT activity (Fig. 2, a), while the control vector J2 lacking the



ψ^+ element appeared to be suppressed in absence of Rex in all three cell lines examined (Fig. 2, b). In Jurkat T-cells, vectors containing the ψ^+ sequence exhibited enhanced Rex independent CAT activity, which tended to further improve by the removal of the CRS element contained in the 5' LTR (Fig. 2, g and h). To localise the critical MoMLV sequences responsible for this stimulatory effect, the ψ^+ sequence was split into four overlapping fragments, by changing the internal *Aat*II and *Bst*EII sites to *Hind*III and cloning each of the four resulting *Hind*III fragments back into J-2. Results from CAT assays indicated that the stimulatory sequence lay at the 3' end of the extended ψ^+ , and was functional in all three cell lines examined. Thus, J-2. ψ 201 and J-2. ψ 560 containing MoMLV nucleotides 165–366 and 165–725 respectively could not promote comparable CAT activities (Fig. 2, c and e). In contrast, J-2. ψ 312 and J-2. ψ 671 containing MoMLV nucleotides 366–1037 and 725–1037, generated CAT activities comparable although somewhat less than the complete ψ^+ sequence (Fig. 2, d and f).

To examine the sub-cellular localisation of CAT transcripts, fluorescent in situ hybridisation (FISH) experiments were carried out. COS, A293, and Jurkat cells transiently transfected with appropriate vectors were fixed and hybridised with specific probes against CAT mRNA under partially denaturing conditions, so as to avoid hybridisation of the probe to DNA [6,24]. Pre- and post-denaturation RNase digestion using 100 μ g/ml RNase A confirmed that FISH signals observed under these partial denaturing conditions were derived from RNA and not DNA (data not shown). In the FISH pictures, RNA is stained green while DNA is stained red (propidium iodide) or blue (DAPI). Yellow staining marks the colocalisation of RNA and DNA. Cytoplasmic RNA was apparently compartmentalised, and therefore did not spread through out the cytoplasm. Depending on the amount of RNA the green (or yellow) staining appeared speckled (low amounts) or diffuse, or uniform (high amounts). This transition was very obvious when the cells were stained in a time course manner after transfection. Similar kinetics and patterns of cytoplasmic distribution were seen in the presence of Rex or upon removal of all CRS elements contained in the HTLV-1 LTR [6].

Typically individual transfected cells showed mainly nuclear or mainly cytoplasmic distributions of CAT mRNA. Depending on the cell type and manipulation of constructs, more or fewer cells show cytoplasmic localisation of CAT mRNA. Tax promoted expression of CAT mRNA from J-2 in both HeLa and A293T cells, but the transcripts remained predominantly nuclear (Fig. 3A, a and Fig. 3B, a respectively). Improved nuclear export of the CAT transcripts was observed with constructs containing the ψ^+ sequence cells (Fig. 3A, d and Fig. 3B, b respectively). By contrast, sequences 5' of the gag open reading frame including the canonical packaging sequence of MoMLV, contained by the construct J-2. ψ .560, failed to promote export (Fig. 3A, c). However, a short fragment of 312 bp overlapping the gag p15/p12 open reading frame efficiently relieved the nuclear entrapment of CAT transcripts (Fig. 3A, b).

The low magnification fields shown in Fig. 3 are characteristic fields, while obviously they are not large enough to be quantitatively reliable. The scoring of multiple fields was done to allow a quantitative analysis of the nuclear cytoplasmic distribution of the mRNAs. The small cytoplasm of Jurkat T cells made it difficult to confidently score these cells with

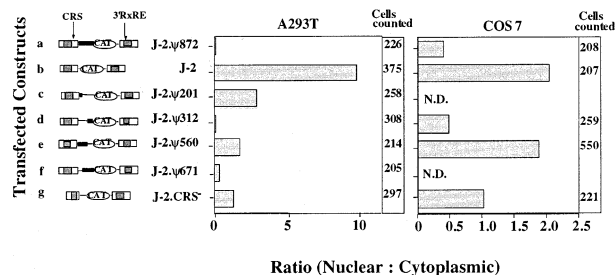


Fig. 4. Quantitation of the nuclear and cytoplasmic distribution of CAT encoded mRNAs. Cells were calcium phosphate transfected with 10 μ g of each CAT expressing plasmid and 2 μ g of Tax expressing plasmids. After 48 h they were subjected to FISH analysis. Nuclear or cytoplasmic staining of CAT encoding mRNAs were visually scored using a fluorescence microscope. The number of counted cells in each instance are indicated on the right hand panel. Ratios of nuclear to cytoplasmic stainings are shown as bar diagrams.

FISH. In the other two cell lines, the frequency of nuclear as compared to cytoplasmic CAT mRNA was visually scored using a fluorescent microscope. The number of cells with predominantly nuclear FISH staining and the number of cells with predominantly cytoplasmic FISH staining were counted for each construct, and the ratio of the two was calculated as a measure of nuclear export.

In agreement with the CAT assays, improved Rex independent nuclear export was observed with constructs containing the complete extended packaging sequence, J-2. ψ 872, or the shorter sequences that included the gag open reading frame, J2. ψ 312 and J2. ψ 671. Cells transfected with these constructs showed a predominant cytoplasmic staining of the CAT encoded transcripts (Fig. 4, a, d and f), while control transfections with J-2 led to predominantly nuclear distribution of CAT mRNAs (Fig. 4, b). This effect was particularly evident in A293T cells, which showed greater differences in response to the MoMLV gag derived sequences than COS7 cells (Fig. 4). Sequences not encoding the gag region produced predominantly nuclear stainings in A293T and mixed distributions of the staining in COS7 cells (Fig. 4, c and e). These studies indicate that sequences overlapping the MoMLV gag p15/p12 open reading frame contain a constitutive RNA nuclear export function, which allows Rex independent expression of transcripts arising from the HTLV-1 based expression vector.

3. Discussion

Adam and Miller [25] provided the first direct evidence for the packaging function of various MoMLV sequences in the absence of other viral sequences; these were designated ψ i, or the extended ψ i+ which includes the gag overlapping sequence. The present report provides evidence for an mRNA export function associated with the extended packaging sequence of the MoMLV. However, particularly in COS7 cells the amount of RNA exported correlated qualitatively but did not correspond quantitatively with the amount of CAT activity measured. That is to say, in the presence of Rex or CTE, there seemed to be much higher CAT activity than expected from the change in the nuclear:cytoplasmic ratio of the CAT mRNA. Rex has been reported to affect not only mRNA transport but also translation and mRNA stability. We expect

that such additional effects would influence the efficiency of CAT expression. We cannot rule out the possibility that CTE may have similar additional effects on expression of the exported mRNAs. The current data are compatible with the previous data on the packaging of retroviral vectors, with the caveat that the authors of the previous work did not consider the possibility that the RNAs containing viral sequences might have different distributions between the nucleus and the cytoplasm, and therefore that the extended packaging signals might simply provide for more efficient nuclear export as opposed to more efficient packaging.

This interpretation of our results is in agreement with recent reports indicating that the HIV-1 Rev/RRE system can increase the expression of avian leukosis virus (ALV) structural proteins in mammalian cells (D-17 canine osteosarcoma) and improve the release of mature ALV virions from these cells. In this system, the Rev/RRE interaction appeared to facilitate the export of full-length unspliced ALV RNA from the nucleus to the cytoplasm, allowing increased production of the ALV structural proteins [26]. The fact that oncogenic retroviruses often retain gag sequences and express transforming proteins that are gag-onc hybrids, encourages a search for similar constitutive mechanism of mRNA transport used by other simple retroviruses.

It is tempting to speculate on a possible role for the characterised 312 nt MoMLV sequence in the regulation of retroviral mRNA splicing. Simple retroviruses lack active viral mechanisms of RNA transport and yet are notorious in simultaneously expressing genes encoded by both spliced and unspliced transcripts. This has led to the assumption, supported by some reports [27,28], that in contrast to transcripts arising from cellular genes splicing of retroviral mRNA is inherently inefficient. Although we cannot rule out additional changes in mRNA stability, the direct examination of subcellular localisation of the transcripts by FISH allows the 312 bp MoMLV gag derived sequence to be defined as a CTE. These findings could provide a new explanation for the apparent inefficient splicing of simple retroviruses as well as the enhanced encapsidation attributed to the extended packaging sequence. Both could be related to a CTE mediated improved nuclear export of viral genomic transcripts. Our observations, however, cannot explain the poor splicing of viral vectors containing only the psi element and lacking the 312 nt sequence, such as the pZip vectors [29]. It should be noted that the efficiency of splicing from these vectors is known to be extremely variable and dependent on the nature of the cDNA insert incorporated into the vector. Nevertheless, these observations suggest that additional factors are likely to contribute to the inefficient splicing of mRNAs of simple retroviruses.

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