The 5’ leader sequence of mouse mammary tumor virus enhances expression of the envelope and reporter genes

Christine Hohenadl,1 Walter H. Gunzburg,1,2 Brian Salmons3 and Stanislav Indik1

1Institute of Virology, Department of Pathobiology, University of Veterinary Medicine Vienna, Vienna, Austria
2Christian Doppler Laboratory for Gene Therapeutic Vector Development, Vienna, Austria
3Austrianova Singapore, Biopolis, Singapore

Mouse mammary tumor virus (MMTV) is a complex betaretrovirus, which utilizes a Rev-like auxiliary protein Rem to export the unspliced viral RNA from the nucleus. MMTV env mRNA appears to be exported via a distinct, Rem-independent, mechanism. Here, we analysed the effect of an extensively folded region coinciding with the 5’ leader sequence on env gene expression. We found that the presence of the 5’ leader stimulates expression of the envelope protein. Enhanced Env production was accompanied by increased cytoplasmic levels of env mRNA. The 5’ leader promotes nucleocytoplasmic translocation and increases stability of env mRNA. The region responsible for this effect was mapped to the distal part of the 5’ leader. Furthermore, the 5’ leader inserted in the sense orientation into a heterologous luciferase expression construct increased luciferase activity.

INTRODUCTION

Viruses developed several strategies to control gene expression in a competitive cellular environment. It has become apparent that regulation of gene expression at the post-transcriptional level, including the control of splicing, mRNA transcript stability, nucleo-cytoplasmic export, subcellular localization and translation, is widespread among viruses. Retroviral RNAs contain several highly structured motifs that modulate various post-transcriptional steps in the expression of viral RNAs. Complex retroviruses such as the prototypic lentivirus human immunodeficiency virus type 1 (HIV-1) and complex betaretroviruses including mouse mammary tumor virus (MMTV), human endogenous retrovirus K (HERV-K) and Jaagsiekte sheep retrovirus (JSRV) carry a cis-acting responsive element that is recognized by virus-encoded Rev-like proteins (Emerman et al., 1989; Felber et al., 1989; Indik et al., 2005a; Löwer et al., 1995; Malim et al., 1988; Mertz et al., 2005; Nitta et al., 2009). The protein–RNA interaction facilitates an efficient nucleo-cytoplasmic transport of the unspliced (singly spliced) viral transcripts via the CRM1/exportin 1 pathway (Fornerod et al., 1997; Fukuda et al., 1997; Neville et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). Simple retroviruses such as Mason-Pfizer monkey virus (MPMV) and simian retrovirus (SRV) lack analogous Rev-like regulatory proteins. Instead, they directly recruit the cellular nuclear RNA export factor 1 (NXF1/Tap) to the structured cis-acting constitutive transport element (CTE) positioned near the 3’ end of viral RNAs (Bray et al., 1994; Zolotukhin et al., 1994). This interaction escorts the nascent viral RNA out of the nucleus and enhances RNA stability. Rous sarcoma virus (RSV) is another simple retrovirus that requires a cis-acting RNA element for nuclear export (Paca et al., 2000). The direct repeats (DR) of RSV are responsible for the accumulation of viral RNA in the cytoplasm (LeBlanc et al., 2007).

The 5’ leader sequence of retroviral RNA has also been reported to be involved in regulation of gene expression. Experiments with spleen necrosis virus (SNV) and later with reticuloendotheliosis virus A (REV-A), human T-cell leukemia virus type 1 (HTLV-1), MPMV, feline leukemia virus (FeLV), human foamy virus (HFV) and bovine leukemia virus (BLV) showed that this region, without affecting steady-state cytoplasmic mRNA levels, functions as a positive post-transcriptional control element (PCE) markedly enhancing translational utilization of mRNAs (Bolinger & Boris-Lawrie, 2009; Bolinger et al., 2007; Butsch et al., 1999; Heinkelein et al., 2000; Roberts & Boris-Lawrie, 2000; Russell et al., 2001). A positive effect of the 5’ leader (in combination with the 3’ LTR) on Env production has been reported for a betaretrovirus closely related to MMTV and JSRV. However, instead of exerting a PCE-like activity, this region positively affected the env mRNA export from the nucleus, enhanced env mRNA
stability and reduced intra-env splicing (Sinn et al., 2005). In addition, another group reported that Env production, in contrast to Gag synthesis, was not attenuated by an inhibitor of CRM1-dependent RNA export pathway, leptomycin B (Nitta et al., 2009). Therefore, these data suggested that the 5′ leader rather than the auxiliary protein encoded by JSRV, Rej (Hofacre et al., 2009), orchestrates the nucleo-cytoplasmic translocation of the env mRNA via a CRM1-independent pathway. That is a pathway distinct from the CRM1-dependent pathway employed by HIV-1 to export the single spliced env mRNA from the nucleus (Emerman et al., 1989; Malim et al., 1989).

A Rev-like protein Rem, encoded by MMTV is essential for cytoplasmic accumulation of the unspliced viral RNA (Indik et al., 2005a; Mertz et al., 2005). However, analogously to JSRV, Rem appears to be dispensable for nuclear export of the env mRNA. Cytoplasmic env mRNA levels were not decreased following either leptomycin B treatment or deletion of the Rem responsive element (RmRE) from the proviral molecular clone (Indik et al., 2005a; Müllner et al., 2008).

The aim of this study was to investigate whether the 5′ leader of MMTV had an effect on gene expression. Using a series of MMTV Env expression constructs and firefly luciferase gene reporter plasmids, we identified a fragment within the 5′ leader located downstream of the R and U5 regions that augmented expression of both MMTV env and firefly luciferase genes. Immunoblot and Northern blot analyses revealed that this region does not exert a PCE-like translational enhancement activity. Instead, the presence of this region increases the cytoplasmic steady-state env mRNA levels and enhances stability of env mRNA.

RESULTS

The presence of the 5′ leader region augments production of MMTV-Env pseudotyped MLV virions

To test whether the 5′ terminal sequences are able to modulate gene expression, a series of MMTV-Env subgenomic expression constructs was generated (Fig. 1a, b). All the plasmids contain the MMTV env gene under the transcriptional control of the heterologous cytomegalovirus (CMV IE) promoter, allowing constitutive, dexamethasone-independent, expression. The expression constructs contain the standard splice donor and acceptor sites for rem mRNA [the spliced rem mRNA is likely exported from the nucleus via the standard pathways for spliced mRNA (Indik et al., 2005a; Mertz et al., 2005)]. MMTV Env expression was first tested using the ability of the MMTV Env to pseudotype MLV particles (Golovkina et al., 1998). We transfected the Env-expression plasmids, together with the MLV-based vector pLXSN–EGFP, into a semi-packaging cell line, 2GP19T, expressing MLV gag and pol genes (Pambalk et al., 2002). Expression of MMTV Env results in the production of EGFP-containing MMTV pseudotypes capable of infecting MMTV-permissive feline kidney cells (CrFK) (Günzburg & Salmons, 1986; Howard et al., 1977; Indik et al., 2005b). Transfection of all four Env expression constructs resulted in infection of CrFK cells (Fig. 1c).

Although transfection efficiencies, measured by FACS analysis of transfected cells, were not significantly different (data not shown), marked differences in the number of transduced cells were detected (Fig. 1c). The lowest level of infection \( \left[ 4.2 \times 10^6 \pm 0.9 \times 10^1 \right] \) green fluorescent units (GFU) ml\(^{-1}\) was obtained with the construct bearing the env ORF alone (Fig. 1c, pEnv). An approximately twofold increase in infection levels was observed with the Env expression construct containing the 5′ leader region (pUTREnv). The presence of the 3′ LTR had a similar positive effect on transduction efficiency (pEnvLTR). The highest increase in virus titres was obtained with the construct bearing both regions flanking the env ORF (Fig. 1c, pUTREnvLTR; \( 3 \times 10^5 \pm 0.8 \times 10^7 \) GFU ml\(^{-1}\). Thus, the results from the pseudotyping/transduction experiments suggest that the 5′ leader and the 3′ LTR have an effect on Env expression.

Differences in pseudotype transduction efficiencies are attributable to differences in Env protein levels

To investigate whether the differences in infection levels are due to differences in Env protein abundance, CrFK cells transfected with the Env expression constructs were subjected to immunoblot analysis. The signal intensities of the obtained bands were densitometrically quantified and normalized to the level of actin protein (Fig. 2a and b). The minimum level of the Env protein is produced in cells transfected with pEnv. When either the 5′ leader or the 3′ LTR were included into the expression plasmids, a substantial increase in amounts of Env was reproducibly observed, indicating that each of these elements has a marked stimulatory effect on protein expression (Fig. 2a, lanes 3 and 4; pUTREnv and pEnvLTR). A further modest (~twofold) increase in Env levels was detected, when both env gene-flanking regions were included in the expression construct (Fig. 2a, lane 5; Fig. 2b, pUTREnvLTR). Collectively, results from the transduction and immunoblot blot analyses indicate that the 5′ leader and the 3′ LTR sequences significantly modulate MMTV Env protein production.

The presence of the 5′ leader increases steady-state env RNA levels

To investigate whether the changes in MMTV Env production could be attributed to changes in steady-state mRNA levels or to an increased translation rate, we performed a Northern blot analysis of cells transfected with the Env expression constructs. Cytoplasmic and nuclear RNAs were isolated 48 h after transfection and analysed.
using an antisense DIG-labelled env probe corresponding to the 5′ region of env ORF (nt 6684–7383). For MMTV, the standard wild-type env mRNA (corresponding to the mRNA expressed from the pUTREnvLTR plasmid) and spliced rem mRNA are 3.8 and 2.6 kb, respectively. Cytoplasmic and nuclear fractionation was monitored using a pre-GAPDH-specific real-time RT-PCR (Blissenbach et al., 2010). The same amounts of extracted nuclear and cytoplasmic fractions were subjected to real-time RT-PCR with primers spanning the intronic sequences of the feline pre-GAPDH. Quantification of the real-time RT-PCR products showed that on average less than 9% (8.6 ± 4.6%) of the extracted cytoplasmic RNA was derived from the nucleus (Fig. 3d). Results from triplicate transfection/Northern blot analyses revealed marked differences in the amounts of unspliced env mRNA. Readily detectable env

**Fig. 1.** Infectivity of MMTV Env-pseudotyped MLV–EGFP virions. (a) Schematic representation of the Env expression constructs used to pseudotype MLV–EGFP particles. All plasmids are based on pcDNA3 (Invitrogen), carrying a CMV IE promoter/enhancer and a bovine growth hormone polyadenylation site (pA). (b) Secondary structure model for the 5′ leader sequence of MMTV. CLUSTAL_X and RNAalifold programs were used to predict the consensus secondary structure model of the 5′ leader. The 5′ leader sequences obtained from GenBank (accession numbers M15122, X00018, X05400, AF228550, AF228552, AF228551 and D16249) were used as input for CLUSTAL_X. The aligned sequences were submitted to RNAalifold web server for the secondary structure prediction. The consensus structure is annotated with positional entropy values (colour code) allowing the identification of conserved structure motifs within the given sequence. High positional entropy values for the stem–loop motifs SL3 and SL4 suggest that this part of the leader is not very stable (in contrast to SL1 and SL2) and may easily adopt an alternative folding. The nucleotide positions are according to BR6 strain (M15122). (c) Supernatants from equal numbers of 2GP19T cells, transiently cotransfected with one of the Env expression constructs and pLXSN–EGFP, were used for infection of CrFK cells. Infectious titres were determined by counting the number of EGFP-expressing cells by FACS. Duplicate infections were performed with equal amounts of supernatant. The data are presented as mean titres (GFU ml⁻¹) and include s.o for each infection. The statistical significance of differences between groups was tested using an unpaired two-tailed Student’s t-test. *, P<0.05.
mRNA was present in the cytoplasmic fraction derived from cells transfected with the wild-type Env expression construct [pUTREnvLTR, Fig. 3(a and b), set to 100%]. In sharp contrast, transfection with pEnv (plasmid lacking the 5’ leader and the 3’ LTR) resulted in less than 7% of env mRNA levels being detected compared with the wild-type transcript (Fig. 3a and b). Markedly reduced cytoplasmic env mRNA levels were also detected with constructs lacking either the 5’ leader (pEnvLTR) or the 3’ LTR (pUTREnv). Quantification of the unspliced env mRNA showed an approximately 65% (pEnvLTR) and 40% (pUTREnv) reduction to that of the wild-type pUTREnvLTR construct (Fig. 3a and b).

Of particular interest were elevated env mRNA levels, resulting from transfections with plasmids containing the 5’ leader. Whereas transfection with the plasmid containing only the Env-coding sequences, pEnv, resulted in the minimum env mRNA levels [6.7 ± 3.3% (average ± sd) of that of the wild-type], presence of the 5’ leader (pUTREnv) significantly elevated cytoplasmic env mRNA levels (Fig. 3a, compare lanes 2 and 3; Fig. 3b, 61.9 ± 3.5% compared with wild-type). Similarly, cytoplasmic fractions of cells transfected with the 5’ leader-containing construct pUTREnvLTR showed a three- to fourfold increase in env mRNA levels over the 5’ leader-lacking construct pEnvLTR (Fig. 3a, compare lanes 4 and 5; Fig. 3b). These results indicate that the 5’ leader has a positive effect on cytoplasmic env mRNA accumulation. Moreover, the presence of relatively high levels of the unspliced mRNA lacking U3R at the 3’ end (pUTREnv) in the cytoplasm and a higher cytoplasmic to nuclear RNA ratio (compared with pEnv, ~fivefold increase) suggests that this mRNA contains a cis-acting element allowing nuclear-cytoplasmic translocation of mRNA.

Interestingly, Northern blot analyses also revealed significant differences in the amounts of the spliced rem mRNA. Cytoplasmic extracts from cells transfected with plasmids containing the 3’ LTR (pEnvLTR and pUTREnvLTR) contained extremely low levels of the spliced mRNA (Fig. 3c). The calculated ratio of unspliced to spliced mRNA was 11.2 for pEnvLTR and 39.5 for pUTREnvLTR (Fig. 3c). Conversely, a significant proportion of mRNA present in the cytoplasm of the pUTREnv- and pEnv-transfected cells represented the spliced rem mRNA (Fig. 3a, lanes 2 and 3). The ratio of unspliced to spliced mRNA was substantially reduced, reaching values of 0.7 and 1.0 for pEnv and pUTREnv, respectively (Fig. 3c). Thus, the presence of the U3R, but not the 5’ leader appears to efficiently interfere with the cellular splicing apparatus.

Based on these Northern blot results we conclude that the 5’- and 3’-terminal sequences have a significant positive effect on the expression of the MMTV env mRNA. Presence of the U3R at the 3’ termini interferes with the activity of the cellular splicing apparatus and allows, to some extent, nucleo-cytoplasmic translocation of the unspliced env mRNA. The 5’ leader sequence of MMTV has a profound effect on the steady-state abundance of cytoplasmic mRNA, suggesting that this region contains a cis-acting element affecting nuclear export of mRNA, although increased stability of env mRNA could also be playing a role. Taken together, our data indicate that the high level of Env protein expression mediated by the pUTREnvLTR construct is not exclusively attributable to an increased translational utilization of env mRNAs.

**The 5’ leader enhances mRNA stability**

To better define mechanism(s) by which the 5’ leader enhances steady-state mRNA levels, we sought to test the stability of env mRNA carrying or lacking the 5’ leader.
sequence. To this end, CrFK cells were transfected with pUTREnvLTR or pEnvLTR constructs. The cells were treated with actinomycin D, an inhibitor of transcription, 48 h post-transfection and subsequently cytoplasmic RNA was isolated for up to 12 h after actinomycin D treatment. Northern blot analysis combined with quantification of the env mRNA showed pronounced differences in stability of the two respective mRNA molecules that differ only in their 5′-terminal sequences. While the env mRNA containing the 5′ leader (pUTREnvLTR) was only minimally degraded 8 h after addition of actinomycin D, the absence of the 5′ leader (pEnvLTR) resulted in a more than 50% decrease in the env mRNA as early as 4 h after actinomycin D treatment (Fig. 4). Therefore, we conclude that the 5′-terminal sequence stabilizes the env mRNA and the observed upregulation of the env gene expression results, at least in part, from this stabilization effect.

**The 3′ end of the 5′ leader enhances gene expression**

To localize the MMTV sequences responsible for the observed enhancement of the env gene expression, we performed a deletion scanning mutagenesis analysis. A
mRNA levels (compared with wild-type pUTREnvLTR) in cytoplasmic extracts from cells transfected with the construct carrying the 5' leader in reverse orientation (Fig. 6b and c). The env mRNA levels determined for this plasmid were similar to the env mRNA levels detected for the construct lacking the 5' leader, pEnvLTR (Fig. 6a and b). Therefore, based on this evidence, we conclude that the 5' leader functions in an orientation-dependent manner to enhance cytoplasmic accumulation of env mRNA.

To further confirm these results, as well as to determine whether the activity of the 5' leader is dependent on the presence of an additional MMTV-encoded factor, we inserted the 5' leader in both orientations upstream of a heterologous firefly luciferase gene. The resulting plasmids pCUTRluc (5' leader in sense orientation) and pCzUTRluc (antisense orientation) were transfected into CrFK cells (Fig. 7) and luciferase activity determined 48 h post-transfection. Results from triplicate transfection experiments showed that the 5' leader in the inverted orientation did not increase luciferase expression compared with a construct lacking the 5' leader, pCMVluc (Fig. 7b). In contrast, a two- to threefold increase in luciferase activity was consistently detected with the plasmid pCUTRluc containing the 5' leader in sense orientation (Fig. 7b). The magnitude of the increase seems to be proportional to the increase observed in Northern blot analyses (data not shown). Therefore, we conclude that the 5' leader per se is sufficient to confer a positive effect on gene expression when placed in the sense orientation.

**DISCUSSION**

The goal of this study was to define whether the MMTV RNA region, previously predicted to be extensively folded and localized within the 5' leader sequence, influences MMTV env gene expression. To this end, the 5' leader sequence was inserted into an MMTV Env expression construct and a luciferase reporter vector, and protein and mRNA levels investigated and compared to controls lacking this region. By these means we demonstrated that the 5' leader has indeed a positive effect on MMTV as well as luciferase gene expression. Both Env production and luciferase activity were increased in the presence of this element placed under the control of the strong CMV IE promoter/enhancer. The magnitude of this increase was proportional to that observed previously for a PCE located in the RU5 region of SNV (Roberts & Boris-Lawrie, 2000). This element stimulated luciferase expression by about 1.7-fold when placed downstream of the CMV IE promoter (Roberts & Boris-Lawrie, 2000). The PCE is a stem–loop RNA structure initially identified within the 5' leader sequence of SNV as a sequence, which has a positive effect on gene expression. A growing number of retroviruses, including MPMV, HFV, REV-A, HTLV-1, FeLV and BLV were subsequently reported to contain a PCE (reviewed by Bolinger & Boris-Lawrie, 2009). Quantitative RNA analysis together with ribosomal sedimentation profile assays

**The 5' leader sequence-mediated effect on gene expression is orientation dependent and the 5' leader augments expression of the non-viral luciferase gene**

To determine whether the 5' leader sequence functions in an orientation-dependent manner, the 5' leader region was inserted in both orientations into the Env expression construct, pEnvLTR (Fig. 6a; sense, pUTREnvLTR; anti-sense, pzUTREnvLTR). Results from two Northern blot analyses show an approximately threefold decrease in env RNA levels (Fig. 4a) and proportional reduction in the amount of env mRNA. Black triangles and squares represent relative RNA levels normalized to the actin mRNA. The series of deletion mutants each lacking 11–70 bp long portion of the 5' leader sequence was constructed (Fig. 5a), the respective plasmids transfected into CrFK cells and cytoplasmic mRNA levels determined by Northern blot analysis. A representative example of three transfection/Northern blot experiments is shown in Fig. 5(b). The mRNA levels (normalized to actin levels) produced by the deletion mutants ΔUTR1321–1370, ΔUTR1371–1415, ΔUTR1415–1470 and ΔUTR1392–1404 were similar in magnitude to the RNA levels produced from the wild-type construct pUTREnvLTR (Fig. 5b, lanes 2–4 and 7; Fig. 5c). In contrast, a marked reduction in mRNA yield was observed for three mutants (ΔUTR1520–1570, ΔUTR1488–1557 and ΔUTR1470–1520) lacking the distal part of the 5' leader in a close proximity to the Env translation start codon (Fig. 5b, lanes 5, 6 and 8; Fig. 5c). In this case, the env mRNA levels were equal to or lower than the levels determined for the UTR-lacking plasmid, pEnvLTR. These results suggest that the 3' proximal end rather than the 5' proximal end and central region of the 5' leader have a positive effect on the cytoplasmic env mRNA steady-state levels.

![Fig. 4. Absence of the 5' leader decreases stability of the env mRNA. Cells transfected with pUTREnvLTR or pEnvLTR, were treated with actinomycin D and the cytoplasmic RNA was extracted at various time points (0, 2, 4, 8 and 12 h) after the treatment. Northern blotting with an env exon-specific DIG-labelled RNA probe was used to detect and densitometrically quantify the env mRNA. Black triangles and squares represent relative RNA levels normalized to the actin mRNA levels detected for pEnvLTR and pUTREnvLTR, respectively.](http://vir.sgmjournals.org)
revealed that this sequence acts at the post-transcriptional level by inducing a robust translation of PCE-containing mRNA (Roberts & Boris-Lawrie, 2000). The translational enhancement is due to the interaction of PCE with the RNA helicase A (Hartman et al., 2006) facilitating polysomal association of mRNA. Analogous to PCE, the 5' leader of MMTV augments gene expression only when inserted in the sense orientation (Fig. 6b and c; Fig. 7b). However, in contrast to the PCE-mediated effect, the present analysis demonstrates a profound effect of the 5' leader on the cytoplasmic mRNA levels. Therefore, we excluded the possibility that the detected increase in gene expression is solely attributable to a translational enhancement. Instead, our experiments suggest that the 5' leader functions as a multimodal region, which affects gene expression mainly at the pre-translational level. In particular, we could show that this region supports nuclear export and enhances stability of the env mRNA transcripts.

The effect of the 5' leader on MMTV mRNA nucleocytoplasmic transport is of particular interest because the native env mRNA has the capacity to encode Rem and contains the RmRE. Therefore, one would anticipate that the Crm1-dependent pathway would be involved in regulation of the cytoplasmic env mRNA levels. However, it appears that Rem and RmRE are dispensable for the extra-nuclear accumulation of the env mRNA. Previously, we showed that unlike the unspliced full-length viral RNA, which is exported from the nucleus only in the presence of Rem/RmRE, deletion of either Rem or RmRE (or both) has only a moderate effect on the export of the env mRNA from the nucleus (Müllner et al., 2008) (unpublished data). Furthermore, treatment of cells with leptomycin B, a drug, which specifically blocks the CRM1-dependent RNA export pathway, markedly reduced cytoplasmic levels of the unspliced viral RNA, while the env mRNA levels remained virtually unchanged (Indik et al., 2005a). These

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**Fig. 5.** Effect of deletions within the 5' leader on the env mRNA expression. (a) Schematic diagram of pUTREnvLTR and its derivatives lacking an 11–70 bp long region within the 5' leader sequence (indicated by a horizontal bold line with Δ symbol). Nucleotide positions are according the BR6 strain (GenBank accession no. M15122). (b) A representative example from three Northern blot analyses of CrFK cells transiently transfected with the Env-expression constructs is shown. The cytoplasmic RNA extracted from the transfected cells was separated on a denaturing agarose gel, transferred onto a PVDF membrane and hybridized to an env exon-specific RNA probe labelled with DIG. Reprobing was performed using an actin-specific RNA probe (bottom panel). NC, Mock-transfected CrFK cells. (c) Densitometric quantification (ImageQuant TL) of env mRNA normalized to actin mRNA levels is shown.
data suggested that the env mRNA, which may be internally spliced (giving rise to the rem mRNA), is able to subvert the normal cellular pre-mRNA processing/splicing machinery and achieves nuclear export via a Rem-independent mechanism. Our results suggest that the 5’ leader is involved in cytoplasmic accumulation of the env mRNA.

A positive post-transcriptional effect of the 5’ leader has previously also been documented for a closely related betaretrovirus, JSRV (Sinn et al., 2005), encoding a Rev-like protein, Rej (Hofacre et al., 2009). Analogously to MMTV, a synergistic positive effect of the JSRV 5’ leader and the 3’ LTR on env gene expression has been reported. Increased cytoplasmic steady-state env mRNA levels resulted from an increased nuclear export, enhanced RNA stability and controlled splicing (Sinn et al., 2005). The combination of these effects ultimately resulted in greater envelope protein levels and higher virus/vector titres. Furthermore, analogously to MMTV, Rej activity has been found to be dispensable for cytoplasmic accumulation of the env mRNA and blocking of the Crm1-dependent RNA export pathway did not inhibit Env expression (Nitta et al., 2009). Therefore, both betaretroviruses appear to employ similar strategies to regulate expression of the unspliced and the single-spliced mRNAs, which differ from that employed by HIV-1. Whereas in the case of HIV-1 Rev mediates nucleo-cytoplasmic translocation of the unspliced as well as the single-spliced mRNAs (Emerman et al., 1989; Malim et al., 1989), Rem (Rej) seems to primarily affect export the unspliced viral RNA.

Our experiments further suggest that, in addition to the mRNA trafficking, the 5’ leader influences mRNA stability.

**Fig. 6.** The 5’ leader enhances gene expression when placed in the sense orientation. (a) Schematic drawing of the pcDNA3-based constructs used in transient transfection assays. The 5’ leader inserted in both orientations into pEnvLTR is depicted as a black pentagon. (b) The Env expression constructs (lanes 1–3) were transfected into CrFK cells. The cytoplasmic RNA was subjected to Northern blot analysis using an env-specific RNA probe together with an anti-DIG antibody and CDP-star chemiluminescent substrate. A representative example of two transfection/Northern blot experiments is shown. An actin-specific RNA probe was used to follow RNA loading (bottom panel). NC, Mock-transfected cells. (c) The env mRNA levels were densitometrically quantified using ImageQuant TL analysis software and percentages of env mRNA relative to pUTREnvLTR are shown. A Student’s t-test was used to evaluate significance of differences between groups. *, P<0.05.

**Fig. 7.** The luciferase activity is modulated by the 5’ leader sequence. (a) Schematic representation of the luciferase expression constructs used for transient transfections. The 5’ leader (black pentagon) was cloned in both orientations into the luciferase expression construct, pCMVluc. The luciferase activity in protein extracts was measured 48 h post-transfection. Results from three independent transfections are shown as mean luciferase activities relative to transfections with a plasmid lacking the 5’ leader, (pCMVluc; set to 100%; so are represented by the vertical bars). The statistical significance of differences between groups was tested using an unpaired two-tailed Student’s t-test. *, P<0.001.
The half-life of RNAs is an intrinsic property determined by their rate of decay and this may be indirectly influenced by mRNA localization. A direct mechanism of RNA degradation commonly involves a sequence- or structure-specific endoribonuclease-recognizing cis-acting cleavage sites. Deletion mutagenesis, used to define sequence boundaries necessary for the 5′ leader activity, identified the distal part of the 5′ leader as the region responsible for the increased gene expression. Thus, we hypothesize that the distal part of the 5′ leader is involved in the interaction with RNA-binding proteins stabilizing mRNA analogous to the previously demonstrated regulation of the transferrin receptor expression (Dodson & Shapiro, 2002). It is also conceivable that the distal part of the 5′ leader adopts a conformation that is more resistant to RNases or drives translocation of mRNA to a cellular compartment lacking (or containing less active) endonucleases (Aragon et al., 2009; Hüttemaier et al., 2005).

The present work provides evidence that the 5′ leader of MMTV increases the steady-state mRNA abundance. Although we cannot rule out the presence of an additional mechanism, we believe that the observed enhancement of gene expression results primarily from a combined effect of the 5′ leader on mRNA stability and nuclear export. The identification of the region responsible for the augmentation of mRNA levels further underlines the importance of the 5′ leader in the life cycle of the prototypic betaretrovirus associated with mammary adenocarcinomas in mice and possibly involved in human breast carcinogenesis and some hepatic disorders (Wang et al., 2004; Xu et al., 2003). This element further broadens the list of functional domains that have been identified within the 5′ leader sequence of betaretroviruses and further emphasizes different strategies that these viruses employ to control gene expression.

METHODS

Cell culture packaging transfections. The human 2GP19T packaging cell line stably transfected with the MLV Gag/Pol expression construct, pGagPolGpt, have been described elsewhere (Pambalk et al., 2002). The CrFK cell line and 2GP19T cells were cultivated in Dulbecco’s modified Eagle’s medium with 10% FBS. Transfections of MMTV-free CrFK cells were performed using the Lipofectamin 2000 transfection kit (Invitrogen Life Technologies) following the manufacturer’s instructions. Briefly, 24 h prior to transfection, cells were seeded at a density of 6 × 10^5 cells per well in six-well plates. The next day, when the cells had reached ~90–95% confluency, transfections with equivalent amounts of Env-expression plasmids (DNA levels of smaller plasmids were adjusted with the basal vector, pcDNA3, to a total amount of 4 μg per reaction) were performed. Transfection efficiency with constructs carrying the Env expression cassette was determined by measuring the luc gene expression after co-transfection of pCMVLuc (0.5 μg, kind gift from Dr Metzner, University of Veterinary Medicine, Vienna, Austria). 2GP19T cells were co-transfected with an MLV-based vector, pLXSN-Egfp (2.5 μg, Klein et al., 1997) and equivalent amounts of the Env expression plasmids (adjusted to 1.8 μg with pcDNA3) by calcium phosphate coprecipitation. Forty-eight hours after transfection, the virus-containing cell culture supernatant was filtered (0.45 μm) and used to transduce 4 × 10^5 CrFK cells seeded in six-well culture plates.

The transduced and transfected cells were analysed by FACS to measure the transfection and transduction efficiencies, respectively. For the luciferase expression analysis, 700 ng pCMVLuc, pCUTRluc and pCUTRLuc were used.

Plasmid construction. The construction of the Env protein expression plasmids pEnvLTR and pEnv has been outlined previously (Indik et al., 2005a; Müller et al., 2008). To introduce the 5′ leader [nt 1321–1608 (position coordinates according to the GenBank sequence M15122)] into pEnvLTR (resulting in pUTREnvLTR) an RT-PCR product [primer pair: 1321FHind (5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′), MCS1321R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1370R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1370R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1370R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1370R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′], pUTREnvK (5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′) corresponding to single-spliced env mRNA was cloned into the HindIII/BamHI sites of pEnvLTR. To make pUTREnv, the same ampiclon was digested with HindIII/XbaI and cloned to pcDNA3. Deletion of 10–70 bp long fragments of the 5′ leader from pUTREnvLTR was obtained by a long template PCR-based approach (Expand Long Range, dNTPack; Roche Diagnostics) using primers pUTR1321–1370 (1370F: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1370R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′), pUTR1371–1415 (1415F: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1370R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′), pUTR1415–1470 (1470F: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1415R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1415R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1415R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1415R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′), pUTR1470–1520 (1520F: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1520R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1520R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1520R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1520R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′), pUTR1392–1490 (1490F: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1490R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1490R: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′), pUTR1488–1557 (1557F: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1557F: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1557F: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′, 1557F: 5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′). Amplification products were digested with EcoRI (underlined) and circularized. Construction of pUTRLuc and pUTRLuc was performed as follows. The 5′ leader flanked by the HindIII restriction sites was generated by high fidelity PCR amplification using the forward primer 1321FHind (5′-AAAAAGAATTCGGGTCTCCCGTCTCGTACTC-3′) and pUTREnv as a template. The HindIII-digested fragment (HindIII is underlined) was then inserted into the corresponding restriction site upstream of the luciferase gene in pCMVLuc. Orientation of inserts was verified by sequencing.

RNA extraction, Northern and immunoblot analyses. For the preparation of the cytoplasmic fraction, the harvested cells (48 h after transfection) were resuspended in 175 μl ice-cold RNl buffer [50 mM Tris/HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 1000 U RNase inhibitor (Qiagen) ml⁻¹, 1 mM dithiothreitol] and incubated on ice for 5 min. The nuclei were pelleted by centrifugation at 300 g for 2 min (4 °C) and the supernatant cytoplasmic fraction was collected and resuspended in 600 μl RLT buffer. To isolate nuclear fractions, the nuclear pellets were washed in PBS and resuspended in 600 μl RLT buffer. Samples were homogenized using QIAshredder spin columns (Qiagen; 2 min, 16 000 g). Cytoplasmic and nuclear RNAs were subsequently extracted using the RNeasy Mini kit according to instructions from the manufacturer (Qiagen). Subcellular fractionation was controlled using real-time RT-PCR (Power SYBR Green RNA-to-CT 1-Step kit; Applied Biosystems) with primers homologous to intron 6 (F-pre- GAPDH fw: 5′-TACCGGGTGATGGGAAAAAG-3′; F-pre-GAPDH rv: 5′-TTCTCCCTGCAGGAGACTG-3′) of the unprocessed pre-mRNA of the glyceraldehyde-3-phosphate dehydrogenase (pre-GAPDH mRNA) (Blissenbach et al., 2010). Extracted cytoplasmic and nuclear RNAs were treated with TURBO DNase (Ambion/Applied Biosystems). Two hundred nanograms of RNA and serial dilutions of the nuclear RNA were
subjected to real-time RT-PCR. Subsequently, percentage of pre-GAPDH mRNA in the cytoplasm relative to nuclear pre-GAPDH mRNA level was calculated. One microgram of extracted RNA was separated on a 1% denaturing formaldehyde gel and blotted onto a Hybond-N+ membrane (GE Healthcare). Following UV-cross-linking and pre-hybridization (for 30 min at 68°C in DIG Easy Hyb buffer; Roche Diagnostics), the membrane was hybridized overnight at 68°C with an env-exon-specific DIG-labelled RNA probe (nt 6684–7383). The RNA–RNA hybrids were detected using an anti-DIG antibody conjugated with alkaline phosphatase, CDP-Star chemiluminescent substrate (Roche Diagnostics) and Amersham Hyperfilm ECL (GE Healthcare). An actin-specific probe was used to monitor for equivalent loading and blotting procedure. Stability of mRNA transcripts was assessed after actinomycin D treatment of CrFK cells transiently transfected with pUTREnvLTR and pEnvLTR, respectively. Actinomycin D (10 μg ml−1 final concentration; Sigma-Aldrich) was added to the transfected cells 48 h post-transfection. The cytoplasmic RNA was extracted from the transfected cells 0, 2, 4, 8 and 12 h after treatment and subjected to Northern blot analysis. The unspliced env mRNA and spliced rem mRNA was quantified using ImageQuant TL analysis software (GE Healthcare) and normalized to actin mRNA levels. Immunoblot analysis was performed as described previously (Indik et al., 2005a).

Luciferase reporter assay. CrFK cells grown in six-well plates were analysed 48 h after transfection using the Bright-Glo Luciferase assay system (Promega). Parallel cultures were assayed for transfection efficiencies by FACS analysis (pCMV-EGFP was co-transfected with luciferase expression constructs). Cells were rinsed once with PBS and disrupted in 500 μl Lysis buffer (5 min, room temperature, rocking platform). Bright Glo assay reagent (100 μl) was added to the same volume of the lysate and luminescence measured using a Tecan GENIOS reader (Tecan Group Ltd). An unpaired two-tailed Student’s t-test was used to determine whether the differences in luciferase activities were statistically significant.

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C. Hohenadl and others


