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Identification of the Rem-responsive element of mouse mammary tumor virus

Matthias Müllner[1,2], Brian Salmons[3], Walter H. Günzburg[1,2] and Stanislav Indik[1,2,*]

[1]Department of Pathobiology, Institute of Virology, University of Veterinary Medicine Vienna, [2]Christian-Doppler Laboratory for Gene Therapeutic Vector Development and [3]Austrianova Biotechnology GmbH, Vienna, A-1210, Austria

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ABSTRACT

Mouse mammary tumor virus (MMTV) has previously been shown to encode a functional homolog of the human immunodeficiency virus-1 (HIV-1) nuclear export protein Rev, termed Rem. Here, we show that deletion of the *rem* gene from a MMTV molecular clone interfered with the nucleo-cytoplasmic transport of genomic length viral mRNA and resulted in a loss of viral capsid (Gag) protein production. Interestingly, nuclear export of single-spliced *env* mRNA was only moderately affected, suggesting that this transcript is, at least to some extent, transported via a distinct, Rem-independent export mechanism. To identify and characterize a *cis*-acting RNA element required for Rem responsiveness (RmRE), extensive computational and functional analyses were performed. By these means a region of 490 nt corresponding to positions nt 8517–nt 9006 in the MMTV reference strain was identified as RmRE. Deletion of this fragment, which spans the *env*-U3 junction region, abolished Gag expression. Furthermore, insertion of this sequence into a heterologous HIV-1-based reporter construct restored, in the presence of Rem, HIV-1 Gag expression to levels determined for the Rev/RRE export system. These results clearly demonstrate that the identified region, whose geometry resembles that of other retroviral-responsive elements, is capable to functionally substitute, in the presence of Rem, for Rev/RRE and thus provide unequivocal evidence that MMTV is a complex retrovirus.

INTRODUCTION

Retroviruses are RNA viruses that replicate their genomic RNA via a proviral DNA intermediate. The synthesis of all viral transcripts takes place in the nucleus of the infected cell and these RNA molecules are subsequently transported to the cytoplasm. As the nuclear export of incompletely spliced RNAs is usually prevented in cells, retroviruses evolved mechanisms that enable circumvention of the cellular splicing machinery by enhanced nuclear export of viral mRNAs.

Simple retroviruses such as Mason-Pfizer monkey virus (M-PMV), simian retrovirus type-1 (SRV-1) and simian retrovirus type-2 (SRV-2) utilize a *cis*-acting viral RNA segment, termed constitutive transport element (CTE), which directly interacts with cellular proteins promoting RNA export from the nucleus. In contrast, complex retroviruses utilize a virally encoded auxiliary *trans*-acting protein, which drives nucleo-cytoplasmic transport of incompletely spliced viral transcripts. For example, the most extensively studied retrovirus, human immunodeficiency virus-1 (HIV-1), encodes a protein termed Rev. Rev interacts with a highly structured viral RNA segment containing several stem–loop structures, the Rev-responsive element (RRE), present in the *env*-coding region (1–4). Nuclear export is mediated by recruiting the karyopherin export factor, chromosome region maintainance 1 (Crm1) protein that directs transport of this complex through the nuclear pores.

Functional homologs of the HIV-1 Rev protein have been described for a number of retroviruses including two members of the genus Betaretrovirus, human endogenous retrovirus K (HERV-K) and mouse mammary tumor virus (MMTV) (5–9).

Interestingly, both Rev-like proteins, Rec of HERV-K and Rem of MMTV, are encoded by doubly spliced transcripts that overlap with the *env* mRNA. The position of the splice donor slightly differs between these two viruses. Whereas the splice donor utilized for the formation of the *rem*-coding sequences is localized within the surface domain (SU) region of the *env* gene, the splice donor of the *rec* sequence is placed within the signal peptide-coding region. Additionally, the *rec*- and *rem*-coding sequences differ in the reading frame of the last exon. While, the reading frame of the entire *rem* RNA is identical to the reading frame of the *env* mRNA, the first exon of the *rec* RNA is spliced into a reading frame different from that of the *env* gene (5,6,9).

*To whom correspondence should be addressed. Tel: +43 1250772333; Fax: +43 1250772690; Email: stanislav.indik@vu-wien.ac.at

Thus, somewhat surprisingly, the *rem*-coding sequence is derived from the entire signal peptide-coding region, in addition to a part of the SU-and transmembrane domain (TM)-coding sequences of the *env* gene.

The responsive RNA sequences to which Rec protein binds (RcRE) were identified and, in contrast to the HIV-1 RRE, shown to be located in the U3-R region of the 3′-long terminal repeat (LTR) of HERV-K transcripts, which has been predicted to form into a complex RNA secondary structure (8,10).

Little is known about the localization of the Rem-responsive element (RmRE). It has previously been suggested that the RmRE is localized near the 3′-end of the MMTV genome. We previously demonstrated that the HIV-1 Rev protein directly binds to the U3 region of the MMTV RNA, suggesting that this segment of the viral genome may represent RmRE (11). It has also been reported that the Rem responsiveness requires the presence of both the LTR and the 3′-end of the *env* gene, suggesting either that the LTR or the *env*-LTR junction may represent the RmRE (5).

Here, we present data showing that the *env*-U3 RNA junction sequence has the potential to form a highly structured region comprising of a long stem followed by a central internal loop from which two short stem–loops protrude, a structure reminiscent of that of previously described for a number of 'responsive elements'. Furthermore, we demonstrate that this segment of the MMTV genome represents the binding site for Rem protein and is required for export of the unspliced viral RNA transcripts.

MATERIALS AND METHODS

Plasmid constructions

For construction of the pCMMTV molecular clone in which the expression is directed by the cytomegalovirus (CMV) promoter, an intermediate plasmid, pCMVenvLTR, harboring the *env* gene and LTR sequence of MMTV (GR strain), was constructed first. The env-LTR fragment was amplified by PCR using high fidelity PCR system (Roche Diagnostics GmbH, Wien), primer 6684FHind (AAA AAA AAG CTT GCC ACC ATG CCG AAT CAC CAA TCT G) in combination with 13574RXba (AAA AAA TCT AGA AGG CTG AGA GGG ACG TA) and the pGR102 MMTV molecular clone (12) as a template. The oligonucleotides introduced HindIII and XbaI restriction sites (underlined) into the PCR product. The PCR conditions were as follows: initial denaturation at 94°C for 2 min followed by 10 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 68°C for 2 min 30 s. Then, 20 cycles consisting of denaturation at 94°C for 15 s, annealing at 55°C and elongation at 68°C for 2 min 30 s plus 5 s for each successive cycle were performed and the reaction finalized by an extension step at 68°C for 7 min. The PCR product was digested with HindIII and XbaI and inserted into the corresponding sites of the pcDNA3 eukaryotic expression vector (Invitrogen, Lofer). The remaining MMTV sequences (R, U5, UTR, *gag* and *pol*) were subsequently introduced into the resulting pCMVenvLTR plasmid. The MMTV sequence spanning positions nt 1321–nt 6890 in the reference MMTV strain (accession number M15122) (13) was amplified by PCR from pGR102. Primers were: 1321FHind (AAA AAA AAG CTT GCA ACA GTC CTA ACA TTC AC) and 6890R (CTC CTC CGC TTC GGA GAT) (HindIII site is underlined). Expand long template PCR system (Roche) was used for the amplification as recommended by the manufacturer for System I. The PCR conditions were: initial denaturation at 94°C for 2 min followed by 10 cycles consisting of three steps (94°C for 10 s, 55°C for 30 s and 68°C for 3 min 30 s) and 20 cycles consisting of three steps (94°C for 10 s, 55°C for 30 s and 68°C for 3 min 30 s extended for additional 5 s elongation for each successive cycle). The amplification was terminated by final elongation at 68°C for 7 min. The amplified sequence was cloned as a HindIII–HindIII fragment into pCMVenvLTR giving rise to a MMTV molecular clone bearing a deletion in the *pol* gene. The 3′-end of the *pol* gene was introduced into the deletion mutant as a SacII–AgeI fragment from the plasmid pGR102. The obtained plasmid was named pCMMTV and sequenced. Analogous to pGR102, the provirus in pCMMTV is a hybrid comprising of sequences derived from endogenous (R-U5-UTR-*gag*) and exogenous (*pol*-*env*-3′-LTR) MMTV sequences. To generate pCMMTV deletion derivatives, the pCMMTV plasmid sequence was amplified using the following primer pairs both carrying an NheI restriction site (underlined): for pCMMTVΔ8546-8954 (dJunLOF: 5′-TAT AGC TAG CCA ATC TAA ACG ATT CGG AGA AC-3′ and dJunLOR: 5′-TAT AGC TAG CCA ATC TAA TGG ATT TAA AGC CTT C-3′), for pCMΔ8663-8838 (dJunSHF: 5′-TAT AGC TAG CCT CTG CTG CAA ACT TGG CAT AGC TCT G-3′ and dJunSHR: 5′-ATA TGC TAG CAG ATC TGA CTG CAC TTG GTC AAG GCT CT-3′), for pCMΔ8697-8947 (dLTR1-250F: 5′-TAT AGC TAG CAA GAT TAC AAT CTA AAC G-3′ and dLTR1-250R: 5′-TAT AGC TAG CTC CCC CTT TTT TCT TTT TT-3′), for pCMΔ8948-9194 (dLTR251-500F: 5′-TAT AGC TAG CAA ATA GAA AGA GAC GCT CAA AAT C-3′ and dLTR251-500R: 5′-TAT AGC TAG CGC ACA GAA GAG TTA TTA TTG AAA GAA TCA AGG G-3′), for pCMΔ9195-9443 (dLTR501-750F: 5′-TAT AGC TAG CCC CTT TCG TGA AAG ACT CG-3′ and dLTR501-750R: 5′-TAT AGC TAG CTC TAT TCC CAT TTC TAA CTT CTG-3′), for pCMΔ9444-9657 (dLTR751-961F: 5′-TAT AGC TAG CAG CCT TTA TTT GCC CAA CCT TGC-3′ and dLTR751-961R: 5′-TAT AGC TAG CGA GGG ATC TGT ATA ACA CTT TAT AGC-3′) and for pCMΔ9658-9893 (dLTR962-1197F: 5′-TAT AGC TAG CGC AAC AGT CCT AAC ATT CTT CTC-3′ and dLTR962-1197R: 5′-TAT AGC TAG CAA TCA TAA TAA CTC ATA CCA TAA GTT TTT GC-3′). Conditions for long template PCR were as follows: initial denaturation at 94°C for 5 min followed by 10 cycles of 94°C for 15 s, 55°C for 20 s and 68°C for 10 min, followed by 20 cycles of 94°C for 15 s, 55°C for 20 s and 68°C for 10 min plus additional 20 s for each successive cycle.

HIV-1 Gag reporter plasmids were constructed from 3-(C) vector kindly provided by H.G. Kräuslich.

This plasmid contains part of the 5′-untranslated region, the complete *gag* gene and the protease-coding region of the *pol* gene of HIV-1 (strain BH-10) as well as the CTE of M-PMV. Expression is driven by the CMV promoter/enhancer and terminated by a bovine growth hormone (BGH) polyadenylation sequence (14). First, to eliminate one of the multiple cloning sites present in the 3-(C), the plasmid was cleaved with Acc65I and EcoRV, the recessive ends filled with T4 polymerase and blunt ends religated. Next, the CTE sequence, present in the resulting intermediate construct (3-(C)Δ), was replaced with the RRE of HIV-1 amplified from the pNLgagSty330 plasmid (kindly donated by B. Felber) resulting in plasmid 3-(RRE) Δ used as a control in experiments demonstrating the responsiveness to *trans*-complementation with HIV-1 Rev. For replacement of the CTE in 3-(C)Δ with potential MMTV-responsive elements, MMTV sequences encompassing nt 8517–nt 9006, nt 8707–nt 8820 and nt 8609–nt 8888 were amplified by PCR using primer pairs EL3 (5′-TAT ATC TAG AGG AGT GAA GGC TTT AAA TCC-3′) and ELJ (5′-TAT ATC TAG ACT GTG GTC CTT GCC CCA AGA-3′); JunBF (5′-TAT ATC TAG ACT GCA GCA GAA ATG GTT GAA CTC C-3′) and JunBR (5′-TAT ATC TAG ATG ATG GGC TCA TCC GTT TGT GC-3′); and StF (5′-TAT ATC TAG ACC CCA TTG TTT TCC AGT GCC TTG-3′) and StR (5′-T AT ATC TAG ATC CCC CAA TAG CCC TAG GCA AAG-3′) (XbaI site is underlined), respectively. pGR102 was used as a template for this amplification which was carried out as follows: an initial denaturation step at 94°C for 2 min followed by cycling program of 94°C for 15 s, 55°C for 30 s and 72°C for 30 s for 30 cycles. A final extension step (72°C for 7 min) terminated the reaction. The PCR products digested with XbaI were inserted in both orientations into the 3-(C)Δ intermediate construct linearized with the same restriction enzyme. The resulting plasmids were termed 3-(eU I)Δ, 3-(eU II)Δand 3-(eU III) Δ, respectively. An additional construct in which the CTE sequence was removed was named 3-(-)Δ. A second copy of the identified minimal Rem-responsive sequence has been amplified using primers 2× F (5′-TAT AGG ATC CCC CCA TTG TTT TCC AGT GCC TTG-3′) 2× R (5′-TAT AGG ATC CT*C CGC GG*T CCC CCA ATA GCC CTA GGC AAA G-3′) (BamHI site is underlined; SacII site is in italic) and cloned into the BamHI site in the 3-(eU III)Δ construct. The third copy of the minimal sequence has been amplified (3× F: 5′-TAT A*CC GCG G*CC CCA TTG TTT TCC AGT GCC TTG-3′; 3× R: 5′-TAT A*CC GCG G*TC CCC CAA TAG CCC TAG GCA AAG-3′) (SacII site is in italic) and inserted in the SacII site of the construct bearing two copies of the minimal sequence. Long template PCR System (Roche) has been employed to delete the stem IIB, IIC or both from the 3-(eU III)Δ plasmid using the following primer pairs dIIBf (5′-ACT TT*C CGC GG*G TCT GCG CAC AAA CGG ATG A-3′) and dIIBr (5′-TGA TA*C CGC GG*C TTG GCT GCT TCT CCC CTA A-3′); dIICf (5′-TGC C*CC CGC GG*A GGG GTT GTT TCC CAC CAA G-3′) and dIICr (5′-ATC T*CC CGC GG*T TTC TGC TGC AGG CGC GGC ATT-3′), dIIBCf (5′-GGGC*CC CGC GG*A TCA GAC AAA GAC ATA CTC ATT C-3′) and dIIBCr (5′-GGG AA*C CGC GG*C CTT TTT TCT TTT TTA AAA GAA GCA-3′). The PCR products were digested with SacII and ligated. The HIV-1 Rev expression construct pCMVsRevBF was derived from the plasmid pCMVsrev (a kind gift from B. Felber) as previously described (11,15). For the generation of a Rem expression construct, the *rem*-coding sequence was amplified from cDNA obtained by reverse transcription of RNA extracted from the MMTV-producing murine cell line GR. The amplified product was inserted as a BamHI–XbaI fragment into the corresponding sites of the pcDNA3 expression vector.

All restriction enzymes were purchased either from New England Biolabs, Germany or Promega, Mannheim. All plasmids were prepared in *Escherichia coli* DH5α (Invitrogen). Before transfections, plasmids were prepared using Plasmid Maxi Kit (Qiagen, Wien).

Cell culture and transfection

The MMTV permissive feline kidney cell line, CrFK, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum (16). Importantly, CrFK cells do not harbor endogenous MMTV sequences which may complicate the interpretation of results. For transient transfections, the Lipofectamin2000 transfection kit (Invitrogen) was used according to the manufacturer's instructions. Briefly, 6×10^5 cells were seeded into each well of a 6-well cell culture plate and cultured overnight. On the day of transfection the cells were 90–95% confluent. The total amount of DNA used for transfections was 4 μg (3-(-)Δ-derivatives; HIV-1 Gag assay) and 8 μg (pCMMTV-derivatives; northern blots; MMTV Gag assay), respectively. Effector plasmid pCMVRem of 2.5 μg (HIV-1 Gag assay) and 4 μg (northern blots; MMTV Gag assay), respectively, was used for *trans*-complementations. Heterologous HIV-1 Gag expression-based assay was established with the RRE-containing reporter plasmid (3-(RRE)Δ) and the Rev expression construct (pCMVsRevBF) (11). In this case, 2 μg of the pCMVsRevBF was used for *trans*-complementations. Equimolar amounts of reporter plasmids were used in all co-transfections. DNA of the plasmid pcDNA3.1 was added as carrier to a final content of 4 μg or 8 μg total DNA, respectively. To monitor the transfection efficiency, 200 ng of an EGFP-expressing construct (pCMVEGFP, Clontech, Saint-Germain-en-Laye) was added to all transfections and the transfection efficiency was monitored by counting the EGFP-positive cells and by immunoblotting using anti-GFP antibody. All transfections were repeated at least three times.

Immunoblotting analyses

Cells harvested 48 h post-transfection were resuspended in lysis buffer (10 mM Tris–Cl [pH 7.5], 140 mM NaCl, 1 mM PMSF, 1% Triton X-100). Total protein extracts of 20 μg were analyzed on 10% polyacrylamide gels containing SDS. Following the electrophoresis, the proteins were electro-blotted to PVDF membranes (Hybond P; GE Healthcare, Wien), the membrane was blocked overnight with 5% nonfat dry milk in TBS-T (20 mM Tris–Cl [pH 7.6], 280 mM NaCl, 0.1% Tween-20) and incubated for 1 h

at room temperature with antiserum against HIV-1 CA (dituted 1:2000; Polymun Scientific Immunobiologische Forschung GmbH, Wien) or MMTV CA (diluted 1:4000 in TBS-T containing 5% nonfat dry milk; kind gift of M. Sakalian). Following washing, the membranes were incubated for 1 h at room temperature with a secondary anti-rabbit antibody conjugated with horse radish peroxidase (diluted in TBS-T 1:10 000; DAKO). Detection was performed using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Wien) according to the manufacturer's instructions. Blots were exposed to X-ray films (GE Healthcare) or the intensity of signals quantified by using a Phosphorimager (Storm 860; GE Healthcare). The membranes were stripped in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–Cl [pH 6.7]) for 30 min at 50°C, blocked and re-probed with antiserum against EGFP (1:500; BD Biosciences, Schwechat) and actin (1:1000, Sigma, Wien).

RNA preparation and northern blot

For preparation of cytoplasmic RNAs, cells were harvested 48 h post-transfection and resuspended in RLN buffer (50 mM Tris–Cl [pH 8.0], 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Igepal CA-630). The cell suspensions were chilled on ice for 5 min and vortexed for 20 s. Nuclei were sedimented by centrifugation at 300*g* for 2 min and isolation of cytoplasmic RNA from the supernatant was performed with RNeasy Mini kit (Qiagen) using manufacturer's protocol. The RNA content was measured photometrically. Cytoplasmic RNA of 1 μg was used for northern blotting. RNA was loaded onto denaturating formaldehyde gels containing 1% agarose, separated at 3 V/cm for 6 h and blotted onto positively charged nylon Hybond N^+ filters (GE Healthcare). After UV-crosslinking, the membrane was pre-hybridized for 30 min at 68°C in DIG Easy Hyb buffer (Roche) and hybridized overnight at 68°C with a specific digoxigenin (DIG)-labeled RNA probe. The probe was prepared by *in vitro* transcription reaction carried out using DIG-11-UTP. As DNA template PCR product synthesized using primer 6684F (5′-ATG CCG AAA CAC CAA TCT G-3′) along with 7383RT (5′-AAT TAA CCC TCA CTA AAG GGC GAT CTG GCA GTG AGG ATA-3′) was used. The *in vitro* transcription reaction was carried out for 1 h at 42°C. Subsequently, DNA template was removed by DNase I treatment. The yield of the DIG-labeled RNA was determined by dot blotting. The MMTV-specific DIG-labeled RNA probe was loaded onto the nylon membrane together with the control DIG-labeled actin RNA probe (Roche). After probing with the anti-DIG antibody conjugated with alkaline phosphatase, and CDP-Star chemiluminescent substrate (Roche), the membrane was exposed to X-ray films (GE Healthcare) and the strength of the RNA signals compared. Stripping and reprobing was performed as follows: the DIG-labeled probe was removed from RNA blots by incubating membranes for 2 h at 80°C in stripping buffer (50 mM Tris–Cl [pH 7.5], 50% formamide, 5% SDS). Following pre-hybridization, membranes were stained with DIG-labeled actin probe (Roche).

Identification of highly structured regions and prediction of RNA secondary structure

The presence of highly structured regions in the MMTV genome was analyzed by a statistical simulation using the program SEGFOLD (17), which assigned significance score (Sigscr) and stability scores (Stbscr) to successive segments along the MMTV sequence. The Sigscr are based on comparisons of the predicted thermodynamic stability of the segment in the window with the mean stability of a large number of randomly permuted corresponding segments. The Stbscr is similarly computed by comparing a real segment at a given place with the average of all others in the sequence resulting from sliding of the fixed size window along the tested sequence. The MFOLD algorithm version 3.2 (18) was then used to calculate the putative secondary structure of the regions identified as highly structured regions.

RESULTS

Computational approach for prediction of putative RmRE location and structure

Previously, it has been suggested that the MMTV RmRE is located near the 3′-end of the MMTV genome, however, the Rem binding site has not been precisely mapped (5,11).

To gain more insight into the location of the RmRE, a two-step approach, which includes computational and mutational analysis, was performed. Given that the previously described 'responsive elements' coincide with tightly folded RNA regions containing up to five short stem–loops atop a long main stem, we first used the same computational approach for the identification of the RmRE that previously allowed the determination of the responsive elements for Rev-like proteins of a number of lentiviruses, deltaretroviruses as well as the Rec-responsive element of another betaretrovirus, HERV-K. (2,19–22). We screened the MMTV genome for the presence of complex RNA secondary structure regions using the SEGFOLD algorithm (10,17,20). The best results were obtained with a window size of 415 nt sliding along the MMTV genome in 1-nt steps. Two highly stable and significantly structured regions were identified (Figure 1A). The highest Stbscr was obtained for a region close to the transcriptional start site. The second highly structured region was found to coincide with the 3′-end of the *env* and the 5′-end of U3, the region which has previously been suggested to bind Rem (5).

Next, we used the MFOLD RNA secondary structure prediction algorithm to predict the likely folding of the 3′-end of the MMTV genome encompassing the identified highly structured region. The structure exhibiting the lowest free energy, which may represent the RmRE, is depicted in Figure 1B. This putative RmRE consists of a long stem with internal loops and bulges folded by annealing residues spanning positions nt 8617–nt 8659 and nt 8840–nt 8888, in the reference MMTV strain BR6 (accession number M15122) described by Moore (13). The stem is followed by a central internal loop from which two stem–loops protrude. The first stem–loop (stem I) is

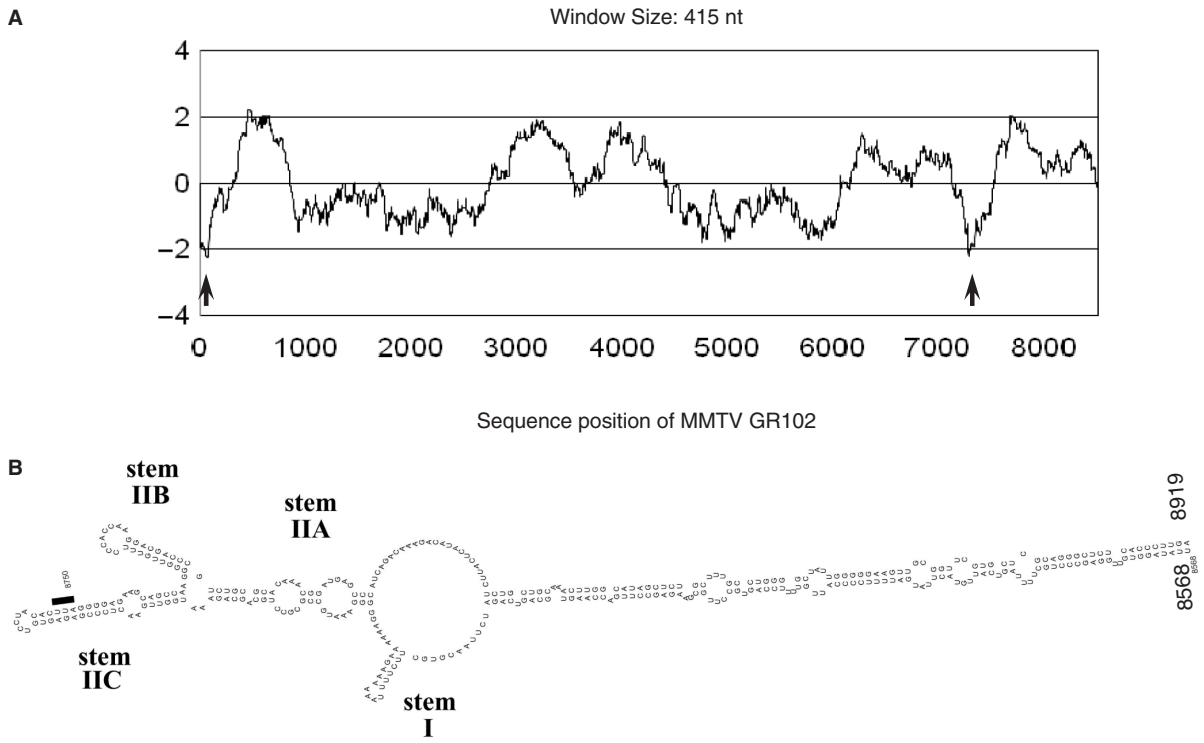

Figure 1. (**A**) Computational detection of highly structured regions in the MMTV GR102 viral RNA. A 415-nt window was moved along the entire viral genome in 1-nt steps and the statistical likelihood of RNA folding was calculated as a Stbscr for each segment. Stbscr is given here as standard deviation units; black arrows denote positions with the highest Stbscr. (**B**) The predicted secondary structure obtained using MFOLD algorithm for the env-U3 junction sequence (corresponding to positions nt 8568—nt 8919). The black bar indicates the stop codon of the *env* gene. The numbers indicate the positions of the respective residues in the BR6 proviral genome (accession number M15122).

short, consisting of only a 6-bp long stem. The second branched stem–loop structure can be subdivided into three stem–loops, stem IIA, stem IIB and stem IIC folded directly at the *env*-U3 overlapping region (Figure 1B).

Deletion analysis of RmRE

Given that most of responsive elements reside in similarly structured regions, consisting of a long main stem with internal loops and bulges, and short stem–loops radiating from a central internal loop (22), we focused our attention on this region of the MMTV genome. In subsequent mutational analyses, a series of viral deletion mutants, including the identified branched stem–loops at the top of the main stem, were constructed from the biologically active proviral clone pGR102 (12) (Figure 2A). The deletion in the first mutant extends from position 7225 (in the *env* gene) to nt 7636 (in the 3′-LTR) within the pGR102 proviral genome, a sequence that is equivalent to residues nt 8546–nt 8954 in the prototypic MMTV strain BR6. Thus, the first deletion mutant was termed pCMΔ8546–8954. In the second mutant, pCMΔ8663–8838, a shorter region also encompassing the *env*-U3 region, corresponding to residues nt 8663–nt 8838 in the reference BR6 strain was deleted. The five remaining mutants, pCMΔ8697-8947, pCMΔ8948–9194, pCMΔ9195–9443, pCMΔ9444–9657, pCMΔ9658–9893, contained successive deletions ranging in length from 210 bp to 250 bp in the 3′-LTR (Figure 2A). Furthermore, a mutant bearing a deletion in the *rem*-coding region was constructed in order to demonstrate an essential function of this protein for expression of viral structural proteins. In all constructs the U3 region in the 5′-LTR was replaced with the human CMV immediate early promoter/enhancer thus obviating the need for initiation of the transcription by addition of a glucocorticoid hormone.

To address the question of whether the introduced mutations exerted an effect on Gag protein expression, which depends on the nucleo-cytoplasmic export of unspliced viral transcript, the MMTV-permissive CrFK cells were transiently transfected with the generated mutant- or wild-type molecular clones. In some of the constructs, the deletion affected the second exon of the *rem* gene. Therefore, to avoid any possible bias due to the presence of an aberrant form of the Rem protein, molecular clones were co-transfected with the Rem-expressing construct pCMVRem. Total cellular extracts from transfected CrFK cells were analyzed by immunoblotting using an anti-MMTV capsid (CA) antibody, specifically detecting the p8-n-CA cleavage intermediate (for simplicity termed Gag). As shown in Figure 2B (lane 2), upon transfection of the MMTV full length molecular clone into CrFK cells, Gag protein was efficiently expressed, whereas no Gag-specific signal was detected in mock-transfected cells (Figure 2B, lane 1). Importantly, the Gag protein was not detected when the *rem*-coding sequence was deleted from the molecular clone

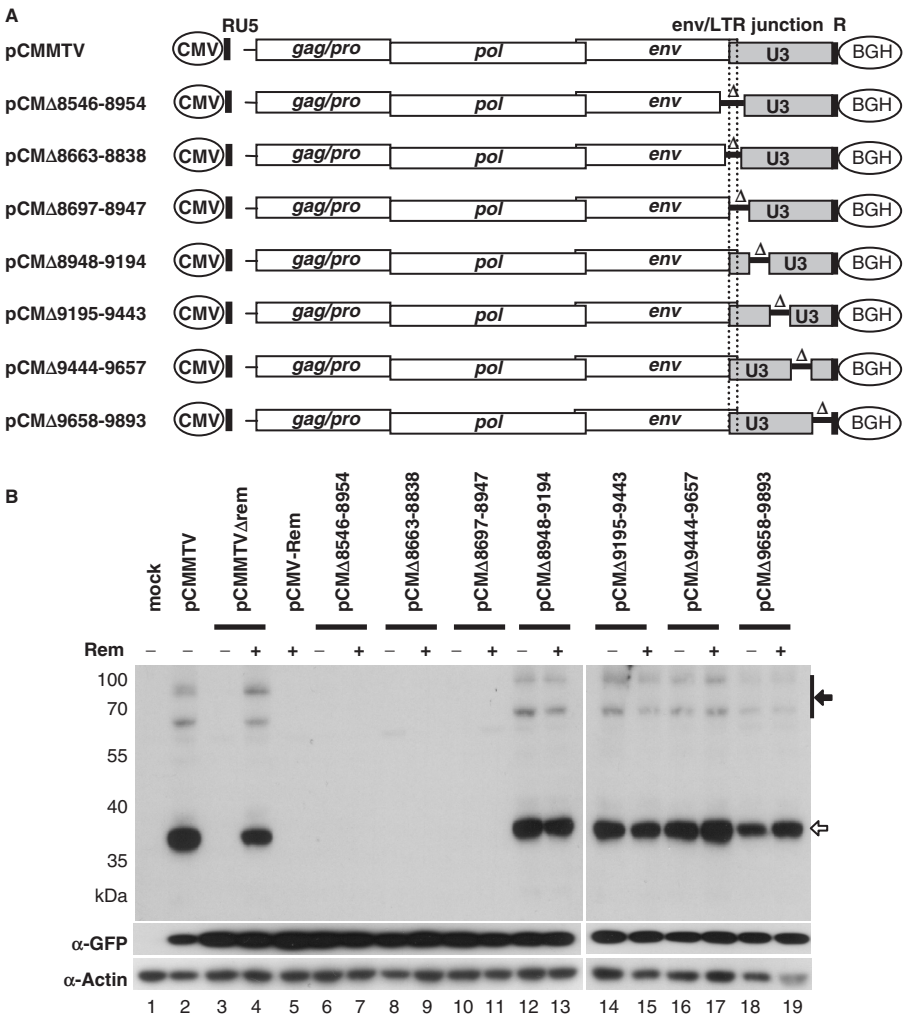

Figure 2. Western blot analysis of cells transfected with MMTV deletion constructs. (**A**) Schematic diagram of the deletion mutants used for transfections. The env-U3 junction is indicated by vertical dotted lines. Deleted regions are indicated by a horizontal bold line; CMV; BGH polyadenylation signal. (**B**) CrFK cells were transiently co-transfected with the expression constructs along with either the Rem effector or empty backbone plasmid. Forty-eight hours after transfection, a crude protein fraction was extracted and analyzed by immunoblotting using an anti-MMTV CA antibody; black arrow indicates Gag polyprotein intermediate products Pr110Gag and Pr77Gag; open arrow denotes p8-n-CA detected by the antiserum. Middle panel: EGFP expression, derived from pCMV-EGFP co-transfected plasmid, was followed using a specific antiserum against EGFP to monitor transfection efficiencies. Bottom panel: to monitor equal loading of the gel and uniformity of blotting, filters were reprobed with an actin-specific antiserum.

(Figure 2, lane 3). The production of Gag was, however, rescued to levels comparable to that of the wild-type pCMMTV vector, when Rem was provided *in trans* (Figure 2, lane 4), thus confirming its essential role for Gag protein expression.

Transfections of the deletion mutants, pCMΔ8948-9194, pCMΔ9195-9443, pCMΔ9444-9657 and pCMΔ9658-9893, yielded approximately the same amount of Gag protein relative to cells transfected with the full length molecular clone, irrespective of the presence or absence of pCMVRem (Figure 2B, lanes 12–19). Importantly, Gag protein was not detected in cells transfected with mutants harboring deletions in the 3′-end of the *env* and the 5′-end of the U3, the region identified by initial *in silico* approach as a potential Rem binding site. A Gag-specific band was not detected in the respective transfected cells even after *trans*-complementation with the Rem-expression construct, thereby ruling out that the lack of Gag production was due to the expression of an aberrant form of Rem (Figure 2B, lanes 6–11). Furthermore, the absence of Gag protein could not be due to reduced transfection efficiencies, since equivalent levels of EGFP protein expressed from pCMV-EGFP plasmid, co-transfected as a control, were observed (Figure 2B, middle panel). Defective Gag production could also not be explained by unequal loading of the gel or a nonuniform blotting procedure, as the same amount of actin was determined after re-probing the filters with an anti-actin antibody (Figure 2B, bottom panel). Overall, these data clearly demonstrate that the sequence at or near the *env*-U3 junction is required for Gag protein expression.

To analyze whether the absence of Gag protein corresponds with a lack of unspliced RNA transcript in the cytoplasm of transfected cells, cytoplasmic RNA was isolated and analyzed by northern blot hybridization using an env-exon-specific probe. Cells transfected with the full length molecular clone, pCMMTV, produced two prominent mRNA species of 8.6 kb and 3.8 kb, corresponding to the unspliced genomic full-length mRNA and single-spliced *env* mRNA, respectively (Figure 3A, lane 1). Unlike the total cellular RNA preparations (Figure 3B, lane 2), the cytoplasmic RNA fraction was devoid of the genomic full-length transcript when the *rem* gene was deleted from the construct (Figure 3A, lane 2). The presence of Rem *in trans* restored the level of the unspliced RNA to approximately the same level as observed with the wild-type molecular clone (Figure 3A, compare lane 1 and lane 3). This clearly showed that Rem is capable of *trans*-complementing the nuclear export defective phenotype of the pCMMTVΔrem mutant.

Next, the LTR deletion mutants were analyzed for the presence of the viral RNA species in the cytoplasm of the transfected cells. As shown in Figure 3A, roughly the same amounts of unspliced RNA relative to pCMMTV were observed for deletion mutants, pCMΔ8948-9194, pCMΔ9195-9443, pCMΔ9444-9657 and pCMΔ9658-9893, lacking sequences downstream of the position 7636 within the pCMMTV construct, a position that corresponds to residue 8947 in the reference strain (Figure 3A, lanes 11–18). Analogous to the immunoblotting results, no cytoplasmic unspliced transcripts were detected in cells transfected with the deletion mutant, pCMΔ8546-8954, lacking 409 bp in the *env*-U3 junction region (Figure 3A, lanes 5 and 6). Gag-encoding RNA was also not detectable in pCMΔ8663-8838 transfectants, in which a shorter deletion comprising of only 176 bp within the region encompassing the end of *env* and the beginning of LTR was introduced (Figure 3A, lanes 7 and 8). Additionally, the genomic-length RNA was also absent in the cytoplasmic fractions of pCMΔ8697-8947 transfected cells (Figure 3A, lane 9 and 10), but was present in total RNA preparations, as for pCMΔ8546-8954 and pCMΔ8663-8838 transfectants (Figure 3B, lanes 5–10). Given that no full-length RNA was detected with these three mutants even in the presence of Rem, together with the fact that control experiments did not reveal unequal transfection efficiency, poor integrity or quality of RNA preparations (data not shown), or uneven loading of the gel (Figure 3A and B, lower panels), we concluded that the introduced deletions affected the predicted RmRE located in this region.

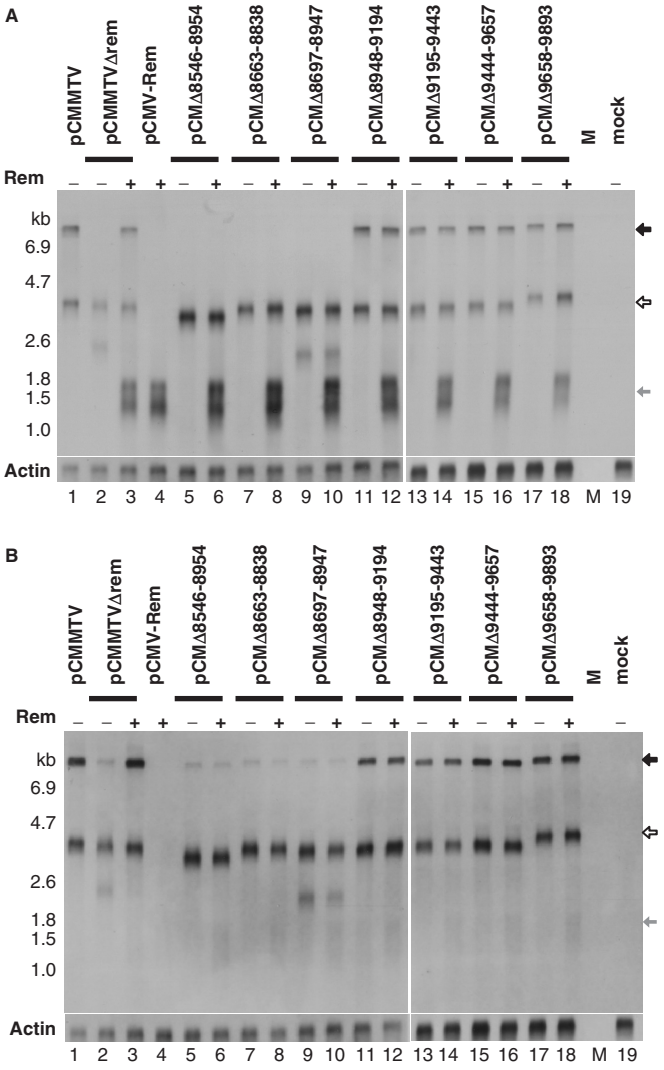

Figure 3. Northern blot analysis of cytoplasmic (**A**) and total (**B**) RNA extracted from the transfected CrFK cells. CrFK cells were transfected with the indicated plasmids; cytoplasmic (A) and total (B) RNAs extracted from the transfected cells and hybridized with an *env*-specific DIG-labeled RNA probe. The membrane was re-hybridized with an actin-specific RNA probe to show equal loading of the gel and the uniformity of the blotting procedure (bottom panel). M: RNA marker; black arrow: unspliced genomic full-length RNA; white arrow: single spliced *env* mRNA; gray arrow: multiply spliced mRNA.

Heterologous reporter system defines sequences essential for Rem responsiveness

To directly analyze whether the *env*-U3 junction region can promote nuclear export of an intron containing RNA, another transient transfection assay, based on HIV-1-derived plasmid 3-(-)Δ was performed (23). This indicator construct contains the HIV-1 *gag*/*pro*-coding sequences sequestered in an intron flanked by a 3′-polylinker that can be used to insert candidate response element sequences. Only if the inserted sequence is able to effectively recruit an RNA export factor, the unspliced *gag*/*pro* mRNA can be exported to the cytoplasm and *gag*/*pro* mRNA-derived protein expression is observed.

First, to demonstrate the functionality of the reporter system, the full-length HIV-1 RRE was introduced into the indicator plasmid [resulting in 3-(RRE)Δ], and responsiveness to Rev was determined (Figure 4A). HIV-1 *gag*-derived protein expression was detected by immunoblotting using antiserum specific for the HIV-1 CA protein. The expression vector for EGFP was co-transfected in all cases to control transfection efficiencies. A readily detected Rev-dependent signal for the precursor polyprotein Pr55Gag, the proteolytically processed CA protein (p24) as well as two intermediate Gag polypeptide cleavage

products (p40 and p49) were observed in 3-(RRE)Δ plasmid-transfected cells (Figure 4B, lanes 6 and 7) as described previously (3,23,24). Virtually no p24 protein and weaker signals for p40, p49 as well as for Pr55Gag precursor were observed without Rev or when the RRE sequence was inserted in an antisense orientation (Figure 4, lanes 6 and 8). No Gag-specific signal was detected in mock-transfected CrFK cells (Figure 4B, lane 1) or in the cells transfected only with the effector constructs pCMV-Rev (Figure 4B, lane 2) and pCMV-Rem (Figure 4B, lane 3), respectively. Accordingly, only a weak signal was observed for *gag*-derived proteins in cellular extracts transfected with the reporter plasmid bearing no responsive element, irrespective of whether Rem was delivered or not (Figure 4B, lanes 4 and 5). Importantly, the reporter construct derived from the parental 3-(-)Δ plasmid by inserting 490 bp of the MMTV provirus which includes the *env*-U3 overlapping region, corresponding to positions nt 8517–nt 9006 in the BR6 strain [3-(eU I) Δ], was indeed responsive to Rem protein. Co-expression of Rem in 3-(eU I)Δ-transfected cells stimulated *gag*/*pro*-derived protein production to levels comparable to that observed for Rev-*trans*-complemented RRE bearing construct allowing easy detection of p24 protein (Figure 4B, lane 7 and 10). This response was dependent on the orientation of the inserted sequence since p24 was not observed in cells transfected with construct carrying the 490–bp long fragment in antisense orientation (Figure 4, lane 11). This data unequivocally demonstrates that the MMTV sequence containing the *env* and LTR overlapping region can, in the presence of Rem, functionally substitute for the Rev/RRE.

The MMTV sequence inserted into the reporter construct is predicted to consist of a long basal RNA stem topped by an RNA loop from which one short stem extends followed by a second longer stem which can be divided into the three subunits (stem IIA, IIB and IIC) (Figure 1B). The question we asked next was whether the short apical structure consisting of the stem–loops IIA–IIC would be sufficient for Rem responsiveness. To this end, a short sequence of 114 bp (nt 8707–nt 8820) was inserted in the 3-(-)Δ indicator plasmid and p24 protein synthesis was, upon *trans*-complementation with Rem, subsequently followed. In the cells transfected with this construct [3-(eU II)Δ no detectable p24 protein expression and only weak production of the Pr55gag polyprotein and its cleavage intermediates was seen (data not shown). We speculate that the unresponsiveness to Rem is a consequence of different folding of the inserted sequence lacking the long stem that would stabilize the structure. Thus, a longer proviral DNA fragment spanning positions nt 8609–nt 8888, including sequences predicted to form the long stem, was inserted into the 3-(-)Δ reporter construct, giving 3-(eU III)Δ. This modification restored Rem responsiveness, as p24 expression could be observed (Figure 4, lanes 12–14). A weaker p24 signal detected with this construct implies that this region may represent a minimal RNA segment required for Rem responsiveness.

Next, we analyzed whether the insertion of multiple copies of the minimal Rem-responsive sequence restores the p24 expression to the levels observed with

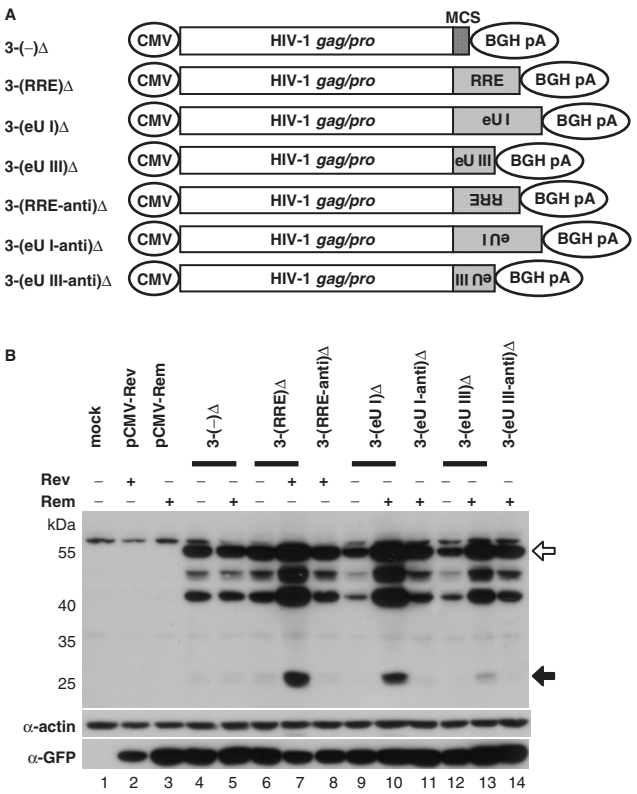

Figure 4. Expression of HIV-1 Gag proteins in cell lysates of CrFK cells transfected with reporter expression constructs. (**A**) Schematic diagram of the indicator plasmids used for co-transfections. The indicator plasmids contain a CMV early promoter/enhancer (CMV), HIV-1 *gag*/*pro* genes, multiple cloning sites (MCS), a RRE, MMTV env-U3 junction sequence (eU I; eU III) and a bovine growth hormone polyadenylation signal (BGH pA). (**B**) Western blot performed with crude extracts of CrFK cells transiently transfected with the indicated plasmids. An HIV-1 CA-specific antiserum was used for immunodetection of the HIV-1 CA protein (black arrow) as well as the Pr55Gag polyprotein (open arrow) and its cleavage intermediates. The product of about 58 kDa detected in all lanes represent a nonspecific cellular protein interacting with the antiserum used for the immunoblotting. The EGFP (bottom panel) and actin (middle panel) proteins were followed to demonstrate equivalent transfection and blotting efficiencies.

the 3-(eU I)Δ construct. Interestingly, the suboptimal Rem responsiveness observed with the minimal construct 3-(eU III)Δhas been improved by inserting the second copy of the minimal sequence in the reporter construct (Figure 5A; 3-(eU III)Δ 2×). Similar levels of p24 protein, comparable to the p24 levels determined with the 3-(eU I) Δ plasmid, have been also expressed in cells transfected with a construct carrying three copies of the minimal sequence (Figure 5A; 3-(eU III)Δ3×).

To determine whether the short apical stems IIB and IIC are crucial for transport of the unspliced RNA, the sequence predicted to fold into the stem IIB, IIC or spanning both stems has been deleted from the 3-(eU I)Δ reporter construct and assayed for the p24 expression. The deletions of stems IIB, IIC or IIB and IIC resulted in complete loss of p24 production, regardless of the presence or absence of Rem, providing further evidence demonstrating that this part of the MMTV genome

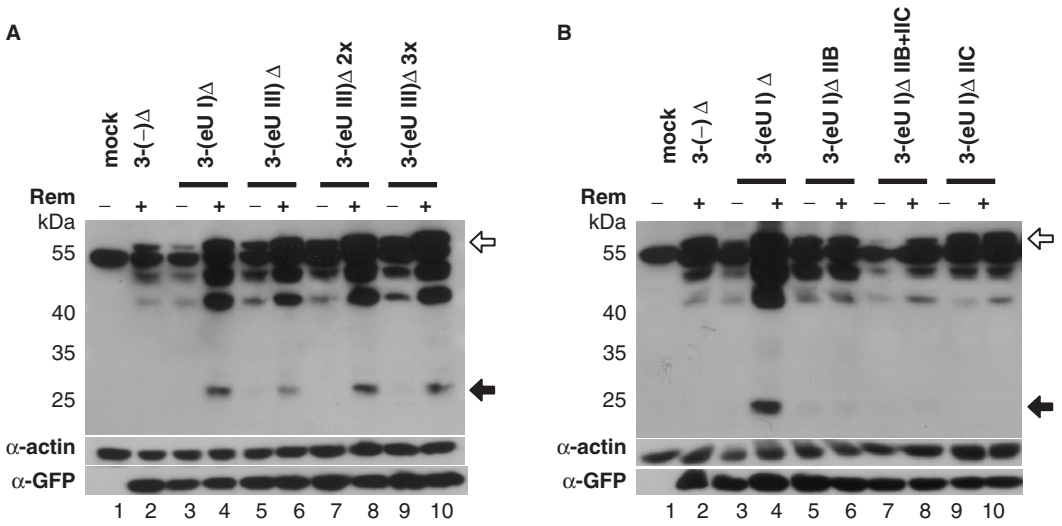

Figure 5. Expression of HIV-1 Gag proteins in cell lysates of CrFK cells transfected with reporter constructs. (**A**) Western blot analysis of CrFK cells transfected with expression constructs carrying one [3-(eU III) Δ], two [3-(eU III) Δ2×] or three [3-(eU III) Δ3×] copies of the minimal RmRE. The p24 expression levels of these constructs were compared with the p24 levels in cells transfected with the 3-(eU I) Δconstruct carrying the 490-bp long RmRE. (**B**) Western blot analysis performed with crude extracts of CrFK cells transiently transfected with the 3-(eU I) Δ derivatives in which the IIB [3-(eU I) Δ], IIC [3-(eU I) ΔIIC] or both (3-(eU I) ΔIIB + IIC) were deleted. An HIV-1 CA-specific antiserum was used for immunodetection of the HIV-1 CA protein (black arrow) as well as the Pr55Gag polyprotein (open arrow) and its cleavage intermediates. The EGFP (bottom panel) and actin (middle panel) proteins were followed to demonstrate equivalent transfection and blotting efficiencies.

plays an important role for the nucleo-cytoplasmic transport of the unspliced RNA (Figure 5B).

DISCUSSION

Several lines of evidence demonstrate that MMTV encodes an ortholog of HIV-1 regulatory protein Rev, termed Rem (5,6). Recruitment of viral Rev-like regulatory proteins to a highly folded RNA 'responsive element' is known to be an essential prerequisite for the biological activity of these factors. Localization of the RmRE of MMTV, however, has not yet been clearly elucidated. It has been shown that the U3 region of the MMTV LTR specifically binds the Rev protein of HIV-1, suggesting that this segment of the viral genome may also represent the binding partner for the Rem protein (11). Another group, however, reported that both the MMTV 3′-LTR and the 3′-end of the *env* gene are required for Rem responsiveness (5). Here, we present data demonstrating that the Rem protein exerts its Rev-like function via binding to a highly structured element, RmRE, encompassing positions nt 8568–nt 8919 (in the reference strain BR6) in the *env*-U3 junction region. This conclusion is supported by several lines of evidence: (i) The identified region is most likely folded into a structure which has a classical responsive element geometry resembling that of many other responsive elements identified so far in that the RmRE structure consists of a long stem which stabilizes the structure topped by two short stem loops. (ii) This region and the *rem*-coding segment are both essential for MMTV Gag protein expression since deletion of either of these sequences leads to a complete loss of Gag protein synthesis. (iii) This lack of expression correlates with the absence of unspliced viral RNA in the cytoplasm but not in total RNA preparations from transfected cells. (iv) The *cis*-acting element, RmRE, accompanied by Rem is capable to functionally substitute Rev/RRE interaction. The Rem/RmRE interaction, similarly to the Rev/RRE interplay, liberates the HIV-1 *gag/pro* mRNA from the effects of cellular proteins that act to retain it in the nucleus, thus facilitating HIV-1 capsid protein expression to similar levels as those seen with Rev/RRE. Importantly, an HIV-1-based reporter construct bearing a smaller region encompassing positions nt 8609–nt 8888 of MMTV was also responsive to *trans*-complementation with Rem (Figure 3. lane13). Lower p24 protein levels detected with this reporter construct suggest that this region may represent a primary Rem binding site. It is well established that the HIV-1 Rev protein functions as an oligomer whereby the Rev monomer initially binds to a high affinity binding site, subsequently triggering the cooperative binding of additional Rev monomers along the structured RRE region (25–34). This process is essential for recruitment of cellular binding partners required for HIV-1 viral RNA nuclear export. Thus, one can envisage that, by analogy with the HIV-1 Rev/RRE, the Rem monomer first binds to the primary binding site, which in turn allows binding of the additional Rem molecules to nearby secondary low-affinity binding sites. The cooperative binding and oligomerization then allows recruitment of cellular factors mediating MMTV RNA transport through nuclear pores. Suboptimal Rem responsiveness obtained with the 3-(eU III)Δ construct therefore suggests that the long stem is required for cooperative addition of consecutive Rem molecules. It could also be possible that the absence of the long stem simply results in misfolding of the MMTV RNA, which in turn does not allow the cooperative binding of additional Rem molecules. An intriguing possibility could also be that the identified

RmRE possesses two primary Rem binding sites, which are both required for an optimal functional interaction with Rem, similarly as has previously been reported for Rec-responsive element of another betaretrovirus, HERV-K, RcRE (10). Two Rec-specific binding sites were found to coincide with two stem–loop subdomains, both of which were required for full biological activity. Thus, it can be speculated that the 3-(eU III)Δ construct carries only one of these two primary Rem binding sites or that one of the sites is aberrantly folded, resulting in only partial restoration of HIV-1 Gag expression. Consistent with this model is the observation that the introduction of the second minimal Rem-responsive sequence restores the HIV-1 Gag expression to the wild-type levels.

Of note is also the finding that the single-spliced *env* mRNA, which can be further spliced to *rem* mRNA, was, in contrast to other retroviruses, only moderately regulated by Rem/RmRE interaction, in accordance with our previous findings (6). Unlike the unspliced viral RNA, the single-spliced *env* mRNA was still observed in cells transfected with the pCMMTVΔrem deletion mutant, albeit at reduced levels (Figure 3A, lanes 1 and 2). Moreover, the *env* transcript was also present, in contrast to the full-length viral RNA, in the cytoplasm of cells transfected with the RmRE deletion mutants (Figure 3A, lanes 5–10). Similar results were obtained using a subgenomic expression construct bearing the *env* cDNA either with a mutated- or wild-type *rem*-coding region. Also, in this case only a moderate decrease of cytoplasmic *env* mRNA levels were observed for the *rem*-deficient mutant compared to cells transfected with the *rem*-expressing construct (data not shown). Taken together, it appears that while the interaction of Rem with the RmRE is essential for cytoplasmic accumulation of unspliced viral transcripts, single-spliced *env* mRNA is, to some extent, able to overcome the cellular pre-mRNA retention machinery and is exported from the nucleus, a property which seems to be unique among retroviruses with complex regulation of gene expression.

MMTV appears to be unique also with respect to the localization of RmRE. The responsive elements have been mapped and characterized for a number of retroviruses with complex regulation of RNA transport including members of the genus *Lentivirus*, *Deltaretrovirus* and *Betaretrovirus*. RREs of lentiviruses are located within the *env* gene, albeit the precise position varies. In most cases, the responsive element spans the region at or near the junction of the SU- and TM-coding regions within the *env* gene (HIV-1, HIV-2, SIV, Maedi-Visna virus, OLV, CAEV) (2,3,21,22,35,36). Two *cis*-acting elements responsible for the post-transcriptional regulation of gene expression through interaction with the Rev-like protein Rex have been identified for the deltaretrovirus HTLV-1. The first Rex-responsive element (RxRE) spans the 5′-splice signal and the second RxRE has been localized within the 3′-LTR (37,38). The 3′-LTR has also been reported to host the responsive element for Rec, an HIV-1 Rev ortholog of the betaretrovirus HERV-K. More specifically, RcRE has been mapped to a 428-nt long region in the U3-R part of the 3′-LTR (8,10). Interestingly, we show here that the responsive element of another betaretrovirus, MMTV (RmRE), consists of a 490-nt long segment spanning the *env*-U3 overlapping region in the 3′-part of the MMTV genome. Thus, it seems that, in contrast to lentiviruses, the location of responsive elements of betaretroviruses considerably varies. Moreover, current classification of betaretroviruses places retroviruses with complex regulation of gene expression (MMTV, HERV-K) into the genus *Betaretrovirus* together with those viruses which utilize the CTE-based mechanism for nuclear export of viral transcripts (M-PMV, SRV-1, SRV-2). Although the mechanism leading to RNA export remains to be elucidated for remaining betaretroviruses [Jaagsiekte sheep retrovirus (JSRV), Langur virus (LNGV) and Squirrel monkey retrovirus (SMRV)] classification of the genus *Betaretrovirus* may require further reconsideration.

In conclusion, we have presented data demonstrating that the RNA sequence located at the junction of the *env* and U3 regions corresponding to positions nt 8517–nt 9006 is very likely folded into a complex secondary structure that is similar to the responsive elements of other retroviruses. Functional analyses showed that this region is indeed the Rem binding site required for the Rem-directed nuclear export of unspliced viral RNA.

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