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Rapid Communication

A novel, mouse mammary tumor virus encoded protein with Rev-like properties

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Abstract

We have identified a novel, multiple spliced, subgenomic mRNA species in MMTV producing cells of different origin containing an open reading frame encoding a 39-kDa Rev-like protein, Rem (regulator of expression of MMTV). An EGFP–Rem fusion protein is shown to be predominantly in the nucleolus. Further leptomycin B inhibits the nuclear export of nonspliced MMTV transcripts, implicating Rem in nuclear export by the Crm1 pathway in MMTV. Rem is thus reminiscent of the Rec protein from the related endogenous human retrovirus, HERV-K.

Keywords: MMTV; Rem; RNA-expression; Regulation; Rec; Rev

Introduction

Mouse mammary tumor virus (MMTV) has been classified as a simple retrovirus, associated with adenocarcinoma and T-cell lymphoma in mice. Unlike other simple retroviruses which have only three open reading frames (ORF) for structural (Gag, Env) and enzymatic (Pol) proteins, MMTV encodes accessory/regulatory factors such as Sag (Acha-Orbea and Palmer, 1991; Marrack et al., 1991) and Naf (Salmons et al., 1990). Moreover, multiple additional promoters have been described in the MMTV genome (Arroyo et al., 1997; Gunzburg et al., 1993; Miller et al., 1992). HERV-K is an endogenous human retrovirus, related to MMTV, and has been recently shown to encode Rec (Lower et al., 1993, 1995), a functional homologue of Rev and Rex of HIV and HTLV, respectively. The 14-kDa Rec protein, encoded by a double spliced RNA, contains a putative arginine-rich nuclear localization signal (NLS) and a putative nuclear export signal (NES), stabilizes incompletely spliced and unspliced viral mRNAs, and enhances their export to the cytoplasm. In contrast to HIV, where the first Rev coding exon is located between *pol* and *env*, the first Rec coding exon comprises nearly the entire signal peptide of HERV-K Env. Interestingly, there is a striking overall similarity between the Env regions of HERV-K and MMTV, both in their hydrophobicity profiles, and in the structure of their respective signal peptides. Moreover, the signal peptides of both HERV-K and MMTV contain an NLS (Hoch-Marchaim et al., 1998; Lower et al., 1995). Here we report the identification of a novel, double spliced MMTV transcript, analogous to Rec that encodes a regulatory protein, Rem.

Results

A novel double spliced MMTV-specific transcript

The MMTV-*env* gene was introduced into the expression vector pcDNA3 between the CMV promoter and the bovine

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growth hormone polyadenylation site to generate an MMTV-Env expression construct. Stable transfection of the resulting plasmid, pCMVenv, into MMTV permissive feline kidney cells, CrFK (Salmons et al., 1985) however, did not result in detectable Env expression, although stable transfection of a plasmid containing the complete MMTV provirus did (Salmons et al., 1985). RT-PCR analysis of mRNAs from pCMVenv transfected cells using primers designed to detect envelope transcripts (Fig. 1A) did not reveal the expected 2 kb fragment corresponding to the full-length *env* RNA, but rather a smaller product of about 0.9 kb was reproducibly detected (Fig. 1B, lane 2). Sequencing of the shorter product revealed that it results from a splicing event. This intra-*env* splicing using the same splice donor and acceptor sites has previously been described in mouse T-lymphoma cells (EL-4) for a Sag encoding transcript initiating from the promoter within the *env* gene (Miller et al., 1992).

PCR using primers located in the CMV promoter and the 3′ end of the *env* gene confirmed the presence of a complete expression cassette in the stably transfected cells (data not shown), suggesting that the vast majority of *env* RNA is spliced in these cells and that this event is responsible for the lack of Env protein expression.

Double spliced transcript is also present in MMTV-infected cells

To determine whether this novel double spliced transcript is biologically relevant, MMTV transcription was analyzed in MMTV producer cells. RNA was extracted from CrFK cells stably transfected with a complete, biologically active, MMTV provirus (2C9), from nontransfected CrFK and from mouse mammary tumor cells (GR) producing large amounts of MMTV (Salmons et al., 1985). RT-PCR using the same primers as above revealed two transcripts in GR and 2C9 cells but not in CrFK cells: the 2-kb product corresponding to the full-length *env* message and the 0.9-kb product resulting from the novel splicing event (Fig. 1B, lanes 3 and 4). In an attempt to identify the initiation site of the double spliced transcript, we performed an RT-PCR with a primer located in the U5 region of the LTR in combination with the same *env* reverse primer (8649R). Two predominant products of 2.3 kb and 1.1 kb were generated (Fig. 1B, lanes 6 and 8) and sequencing confirmed that the longer product represents the subgenomic *env* RNA transcript and the smaller fragment arises from a double spliced RNA species encoding an open reading frame. The intra-*env* splicing was identical to that in pCMVenv transfected cells.

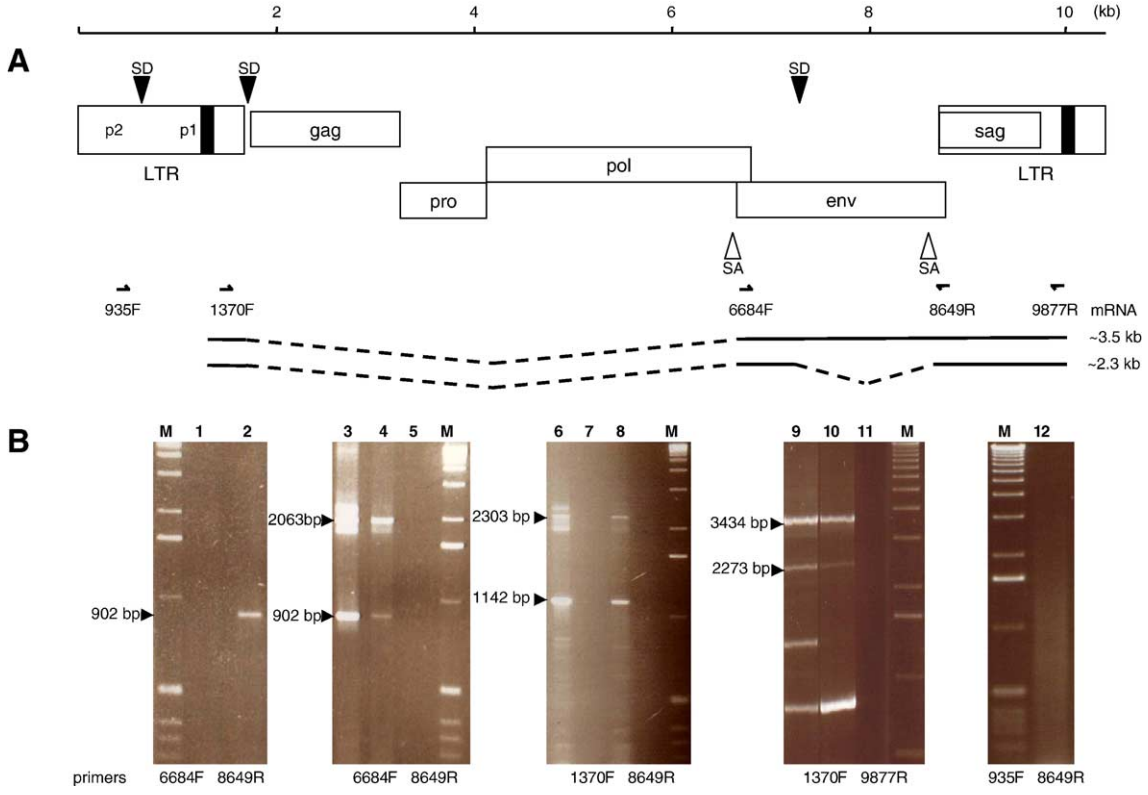

Fig. 1. (A) Schematic representation of the MMTV proviral genome is shown at the top. Open triangles indicate the locations of known splice acceptor sites, and closed triangles indicate the locations of the splice donor sites. The arrows represent the locations and orientations of the oligonucleotide primers used in RT-PCR. Using these primers a doubly spliced RNA species, in addition to env RNA, was identified. (B) RT-PCR was performed with primers 6684F and 8649R (lanes 1, 2, 3, 4, and 5); 1370F and 8649R (lanes 6, 7, and 8); 1370F and 9877R (lanes 9, 10, ans 11); 935F and 8649R (lane 12). RNA used for RT-PCR was extracted from CrFK cells (lane 1, 5, 7, and 11); CrFK cells transfected by pCMVenv expression vector (lane 2); GR cells (lanes 3, 6, 10, and 12) and 2C9 cells (4, 8, and 9). The sequencing of the product extracted from the agarose gel confirmed specificity of the reaction.

The novel RNA species is not further spliced in the 3′LTR region since a specific product of just under 2.3 kb was detected by RT-PCR performed with the U5 primer in combination with a reverse primer which binds 7 nt upstream of the MMTV polyadenylation signal (Fig. 1B, lanes 9 and 10). No RT-PCR products could be detected using a forward primer located immediately downstream of the P2 cap site (Gunzburg et al., 1993) in combination with the *env* reverse primer (Fig. 1B, lane 12). Taken together these results suggest that the novel double spliced RNA originates from the classic P1 promoter (Fig 1B).

The first exon of the double spliced RNA is noncoding and is shared with *env* RNA, while the second exon comprises the entire signal peptide coding region and part of the SU domain of Env. The third exon is common to the end of the *env* mRNA (Fig 1A). This complex splicing pattern is reminiscent of the splicing pattern of mRNA coding for regulatory proteins in complex retroviruses, especially HERV-K Rec (Lower et al., 1993).

The size of the double spliced viral RNA corresponds to that previously detected by several groups in MMTV-infected cells. Northern blots of RNA from virus-induced mammary tumors in high tumor incidence strains of mice revealed a message estimated as 2.5 kb after hybridization with an *env* probe. The message contained LTR and leader sequences and was proposed to be likely a splicing product (Robertson and Varmus, 1981). A 2.5-kb MMTV RNA species was also detected in MMTV-infected rat XC and GR cells in addition to viral, nonspliced, genomic transcript, and major subgenomic *env* RNA (Wheeler et al., 1983). Moreover, an analogous doubly spliced RNA species was detected in EL-4 T-cell lymphoma cells (Racevskis, 1990).

Novel 39-kDa protein encoded by MMTV

The newly identified ORF generated by the doubly spliced transcript is 906 nt long and can potentially encode a 34-kDa protein (Fig. 2A). Specific antiserum was raised by immunizing rabbits with a synthetic peptide (QRPHLALRRKRRREC) corresponding to a region from the hydrophilic highly charged N-terminal part of the putative protein. This polyclonal antiserum was then employed to investigate expression of the putative protein in vitro. A 39-kDa protein was detected specifically in protein extracts from MMTV producing cells (2C9 and GR) but not from their non-MMTV producing counterparts (CrFK and NMuMG, respectively) (Fig. 3A). The presence of two potential asparagine-linked glycosylation sites (Fig. 2A) allowing the attachment of two oligosaccharide units (each with an average molecular mass of 2.5 kDa) to the apoprotein may cause slower migration in the gel, giving rise to a slightly larger protein (39 kDa) than that predicted from the amino acid sequence (34 kDa).

MMTV 39-kDa protein carries putative NLS and NES motifs

Further analysis of the predicted amino acid sequence of the 39 kDa protein revealed the presence of a highly basic arginine-rich N-terminal region, which resembles the NLS identified in the prototype lentiviral transactivator Rev of HIV-1 (Malim et al., 1989) (Figs. 2A and C). This highly basic region confers both specific RNA binding and nucleolar localization to Rev. Another hallmark of the HIV-1 Rev is the presence of a small leucine-rich motif (nuclear export signal, NES) referred as to the Rev effector or activation domain. This domain mediates the interaction between Rev and the cellular cofactor, Crm1, thereby directing export of Rev from

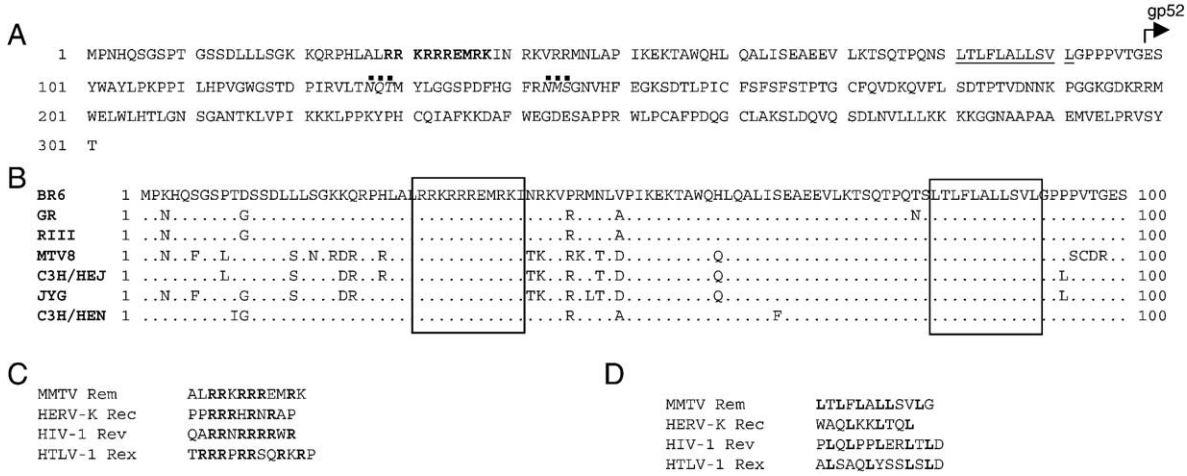

Fig. 2. (A) Deduced amino acid sequences of the putative protein encoded by the double spliced RNA. A putative NLS is marked in bold and the putative NES is underlined. The beginning of the MMTV SU is indicated by an arrow and gp52. Two potential N-glycosylation sites (NQT and NMS) are indicated by italics with dots above the letters. (B) Alignment of the first 100 amino acids of the putative protein encoded by the double spliced RNA; dots indicate the same amino acid as the reference strain BR6. Conserved putative NLS and NES domains are indicated by the two rectangles, respectively. (C) Comparison of the putative arginine (R in bold) rich NLS of the MMTV protein encoded by the double spliced RNA with the NLS of HIV-1 Rev, HTLV-1 Rex, and HERV-K Rec. (D) Putative leucine (L in bold) rich NES of the MMTV protein encoded by double spliced RNA compared to the NES of the Rec, Rev, and Rex proteins.

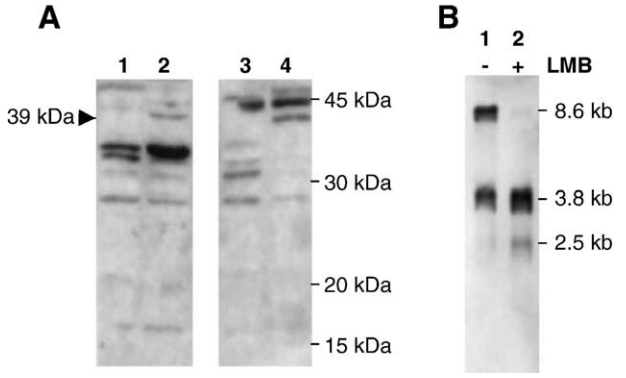

Fig. 3. (A) Western blot analysis of MMTV-bearing cells with an anti-peptide antibody raised against the N-terminal arginine-rich region (putative nuclear localization signal) of the protein encoded by the double spliced transcript. NMuMG (lane 1), GR (lane 2), CrFK (lane 3), and 2C9 cells (lane 4) were stimulated with dexamethasone and expression of the protein encoded by the double spliced RNA was detected. (B) Effect of LMB treatment on export of MMTV transcripts from the nucleus. GR cells were stimulated with dexamethasone and either treated or not treated with LMB, cytoplasmic RNA prepared and analyzed by Northern blotting using an MMTV-LTR-specific probe. Cytoplasmic RNA from MMTV producing GR mammary cells not treated (lane 1) or treated (lane 2) with LMB are shown.

the nucleus (Malim et al., 1991). A similar leucine-rich domain was also identified by analyzing the amino acid sequence of the 39-kDa MMTV protein encoded by the novel double spliced RNA (Figs. 2A and D). Both the putative NLS and NES motifs are highly conserved between various strains of MMTV (Fig. 2B) suggesting their functional relevance. The closely related beta-retrovirus HERV-K also exhibits a complex splicing pattern and the Rec protein, encoded by a double spliced RNA, carries NLS and NES consensus sequences suggesting that this protein shuttles between the nucleus and cytoplasm of the cell. We propose that the 39-kDa protein we have identified is the MMTV homolog of HERV-K Rec and thus HIV-1 Rev and HTLV Rex. Therefore we have called this protein Rem for regulator of expression of MMTV.

A fusion EGFP–Rem protein is localized within the nucleoli

To investigate intracellular distribution of Rem, cells were transfected with the plasmid pEGFP–Rem encoding Rem fused to the enhanced green fluorescent protein (EGFP). The EGFP–Rem fusion protein was predominantly localized in the nucleoli of cells (Fig. 4, panel F), but minor levels of the fusion protein were also present in the cytoplasm. In contrast, cells transfected with an EGFP expression vector (pEGFP–C1) without the Rem fusion (Fig. 4, panel B) or with a fusion plasmid encoding Rem in the antisense orientation (data not shown) showed a cytoplasmic distribution of EGFP. Immunocytochemistry with a red-fluorescence-coupled antibody directed against nucleophosmin (also known as nucleolar phosphoprotein B23) was performed (Fig. 4, panels C and G). Overlay of these images with the corresponding EGFP data reveals the colocalization of EGFP–Rem (Fig. 4, panel H) but not EGFP (Fig. 4, panel D) with nucleophosmin thereby confirming a mainly nucleolar localization of the fusion protein. Together, these data confirm that Rem contains a functional NLS and that its subcellular nucleo-cytoplasmic localization is similar to the localization of the Rec and Rev proteins (Lower et al., 1995; Wolff et al., 1997). The predominant nucleolar localization of the EGFP–Rem fusion protein is reproducibly observed in various murine (NMuMG), human (293, HeLa), and feline (CrFK) cells (not shown), suggesting that this localization is not a cell-specific property.

Export of MMTV nonspliced, genomic RNA is Crm1 dependent

The Rec protein of HERV-K, like Rev of HIV-1, mediates the export of nonspliced viral RNAs through its interaction with the nuclear export factor, Crm1. This interaction can be specifically blocked by the antibiotic

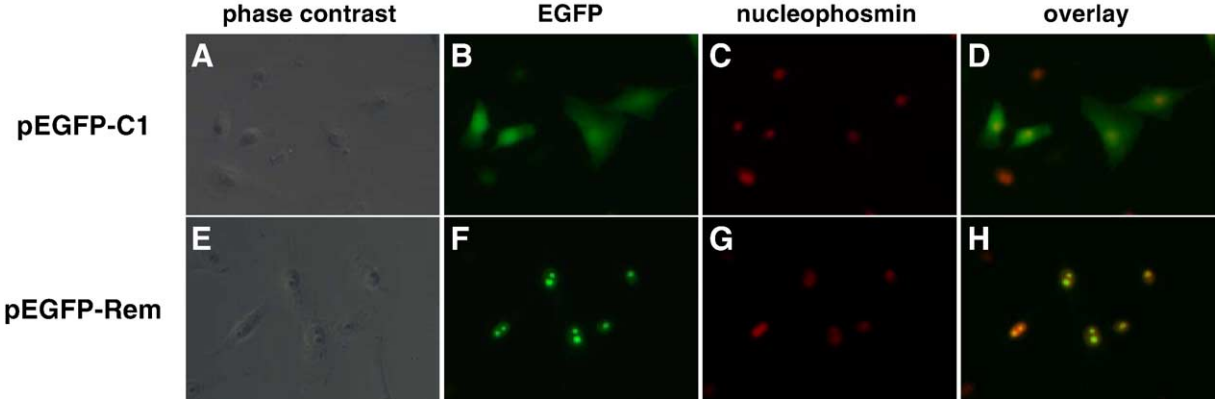

Fig. 4. Intracellular distribution of the EGFP–Rem fusion protein. CrFK cells were transfected with either pEGFP–C1 (panels A–D) or pEGFP–Rem (panels E–H) encoding an EGFP–Rem fusion protein and nucleoli were visualized by indirect immunostaining with anti-nucleophosmin antibody followed by RPE-conjugated secondary antibody. Shown are images of the transfected cells under phase contrast (panels A and E), UV to reveal EGFP fluorescence (panels B and F), or RPE fluorescence (panels C and G) as well as an overlay (merged image) of EGFP and the nucleophosmin images (panels D and H). Magnification is 400×.

leptomycin B (LMB), which traps the Rec or Rev protein in the nucleus and thus inhibits export of nonspliced RNAs (Wolff et al., 1997). To investigate whether export of nonspliced MMTV RNA also proceeds through an LMB sensitive Crm1-dependent pathway, GR cells were treated with the antibiotic, cytoplasmic RNA harvested 14 h later, and a Northern blot was performed. Non-LMB-treated GR cells produce a 8.6-kb nonspliced transcript as well as the 3.8-kb env RNA and the 2.5-kb transcript, corresponding to RT-PCR product in Fig. 1. Treatment of these cells with LMB results in a loss of detection of the 8.6-kb nonspliced transcript and an increase in the levels of the 2.5-kb transcript. These data further support the notion that export of nonspliced MMTV mRNA from the nucleus is Crm1 dependent.

Discussion

In this communication we demonstrate that expression of MMTV from GR and 2C9 cells results in a complex pattern of mRNA species including a novel doubly spliced transcript with an ORF with the potential to encode for an auxiliary protein containing amino acid motifs which resemble the nuclear localization and export signals of the viral regulatory proteins Rev of HIV and Rec of HERV-K.

Using an antiserum raised against a peptide from the putative hydrophilic region of the predicted protein, a 39-kDa protein was observed in two MMTV-producing cell lines (GR and 2C9). To our knowledge, this protein is larger than all known Rev-like proteins (for instance 14 kDa Rec of HERV-K, 19 kDa Rev of HIV-1, and 27 kDa Rex of HTLV-1). Hoch-Marchaim and coworkers also raised an antiserum against the same peptide from the leader peptide region and detected two smaller nucleolar proteins, p14 and p21, specifically in MMTV-bearing T-cell lymphomas but not in MMTV-infected mammary tumors or derived cell lines (Hoch-Marchaim et al., 1998). Similar to these authors, we could not detect p14 or p21 in GR cells which are derived from an MMTV induced mammary carcinoma or the productively transfected 2C9 cells (Salmons et al., 1985). At present, it cannot be excluded that the 39-kDa protein detected in both GR and 2C9 cells is further processed and what effect, if any, this would have on its activity. In this light, it is intriguing that p14 expressed in a several MMTV-derived T-cell lymphomas co-immunoprecipitated and co-localized with the nucleolar phosphoprotein B23, a protein known to interact with HIV Rev and HTLV Rex proteins (Hoch-Marchaim et al., 2003).

Analogous to Rev or Rec, Rem is expressed from a doubly spliced viral transcript and possesses a putative NLS that mediates nuclear import which is inextricably linked with nucleolar localization (Hope et al., 1990). Here we demonstrate that Rem fused to cytoplasmic EGFP is predominantly localized in the nucleolus, indicating efficient nuclear import of the fusion protein and accumulation in the nucleoli. Similarly, nucleolar accumulation of Rec and Rev was indeed reported for HERV-K and HIV, respectively (Lower et al., 1995; Wolff et al., 1997). In analogy to Rev and Rec, low levels of Rem were also present in the cytoplasm.

Another characteristic feature of retroviruses that regulate nuclear export of viral RNA via the Crm1 rather than the Tap1 pathway is sensitivity to inhibition by LMB. This low-molecular-weight antibiotic specifically blocks NES-dependent RNA export. In contrast, cytoplasmic accumulation of RNA containing the constitutive transport element (CTE) of Mason–Pfizer monkey virus or another *cis*-acting RNA export element, termed the posttranscriptional regulatory element (RTE), is not inhibited by LMB treatment (Otero et al., 1998). We hypothesized that although Rem contains a consensus NES, its function requires Crm1 and thus the nuclear export of nonspliced MMTV RNA would be inhibited by LMB. Indeed, LMB treatment of MMTV producing mammary cells resulted in an almost complete loss of nonspliced transcript in the cytoplasm of these cells. To our knowledge this is the first demonstration that the nuclear export of the MMTV full-length transcript requires the Crm1 pathway.

The finding that MMTV encodes a novel protein, Rem, associated with post-transcriptional RNA export adds yet another level of complexity to this betaretrovirus. It remains to be determined what effect, if any, Rem has on spliced transcripts such as those encoding Sag or Naf.

Materials and methods

Plasmid constructions

The plasmid pCMVenv was constructed by inserting the env ORF into the *Hin*dIII–*Xba*I digested expression vector plasmid, pcDNA3 (Invitrogen). The plasmid pEGFP–Rem was constructed by inserting full-length ORF encoded by double spliced transcript in-frame into the *Bam*HI-digested pEGFP–C1 vector (Clontech). The same ORF was also inserted into pEGFP–C1 vector in anti-sense orientation, creating pEGFP–Rem-anti plasmid.

Transfections

Transfection of 3 μg of the respective plasmid was performed in six-well plates using the calcium phosphate method. Stably transfected cells selected in medium containing 400 μg/ml were used for further analysis.

Western blot

Cell monolayers were washed with cold PBS and lysed in buffer (10 mM Tris–HCl, 140 mM NaCl, 1% Triton X-100, 1 mM PMSF). The lysate was clarified by low speed centrifugation and protein concentration measured (Bio-Rad DC Protein assay). 20 μg of total protein was separated on

SDS–polyacrylamide gels and transferred to PDVF membranes. Antipeptide serum raised against the peptide QRPHLALRRKRRREC (Genosphere Biotechnologies) was used for detection of the Rem protein in cell lysates.

Cytoplasmic RNA extraction and Northern blot

Expression of MMTV proteins was stimulated by adding 10^{-6} M dexamethasone and at the same time 10 nM leptomycin B was added to the culture medium for 14 h. Cytoplasmic RNA was then extracted (using an RNeasy Mini kit, Qiagen) according to the manufacturer's instructions and 3 μg applied to a 1.2% agarose gel. After blotting onto a nylon membrane, the filter was probed with a DIG-labeled MMTV-LTR-specific RNA. The probe was prepared by in vitro transcription of the PCR product using a Roche DIG Northern starter kit generated using the following primers (8698F: 5′-ATGCCGCGCCTGCAGCAGAA-3′ and 9400RT3: 5′-AATTAACCCTCACTAAAGGGACACCAAGGAGGTCTAGCTCTG-3′).

RT-PCR

Total cellular RNA was used as a template for RT-PCR. Reverse transcription with MMTV-specific reverse primer (8649R: 5′-GTGTAGGACACTCTCGGGAGTTC-3′, 9877R: 5′-TCAGCACTCTTTTATATT-3′) was performed according to manufacturer's instructions (Superscript II Reverse Transcriptase, Invitrogen). In the next step, RNA complementary to the cDNA was removed by RNaseH digestion and PCR was carried out with the same reverse primer in combination with appropriate forward primer (935F: 5′-AAGACGACATGAAACAACAG-3′, 1370F: 5′-CGTCTCCGCTCGTCACTTAT-3′, 6684F: 5′-ATGCCGAAACACCAATCTG-3′). Specificity of the PCR was confirmed by direct sequencing of PCR products in both directions.

Fluorescence microscopy

CrFK cells transfected with pEGFP–C1 or pEGFP–Rem plasmid were fixed by 2% formaldehyde in PBS for 5 min and permeabilized in 0.2% Triton X-100 in PBS for 20 min. Then, cells were blocked in PBS containing 1% BSA for 1 h at room temperature and immunostained with anti-nucleophosmin monoclonal antibody (Sigma) for 1 h at room temperature followed by incubating with RPE-conjugated anti-mouse secondary antibody (Dako) for another 1 h. After mounting, cells were observed in a Carl Zeiss Axiovert 200M UV microscope.

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