Nuclear RNA Export and Packaging Functions of Rev

Nuclear RNA Export and Packaging Functions of

HIV-1 Rev Revisited

Maik Blißenbach, Bastian Grewe, Bianca Hoffmann, Sabine Brandt, Klaus Überla*

Department of Molecular and Medical Virology, Ruhr-University Bochum, Germany

Running title: Nuclear RNA Export and Packaging Functions of Rev

Abstract: 153 words
Whole text: 7043 words

*Correspondent footnote:
Department of Molecular and Medical Virology
Ruhr-University Bochum
D-44780 Bochum, Germany
Tel.: 0049-234-3223189
Fax: 0049-234-3214352
E-mail: klaus.ueberla@rub.de
Nuclear RNA Export and Packaging Functions of Rev

Abstract

Although the viral Rev protein is necessary for HIV replication, its main function in the viral replication cycle has been controversial. Re-investigating the effect of Rev on the HIV-1 RNA distribution in various cell lines and primary cells revealed that Rev enhanced cytoplasmic levels of the unspliced HIV-1 RNA mostly 3- to 12-fold, while encapsidation of the RNA and viral infectivity could be stimulated more than 1000-fold. Although this clearly questions the general notion that the nuclear export of viral RNAs is the major function of Rev, mechanistically encapsidation seems to be linked to nuclear export, since the tethering of the nuclear export factor TAP to the HIV-1 RNA also enhanced encapsidation. Interference with the formation of an inhibitory ribonucleoprotein complex in the nucleus could lead to enhanced accessibility of the cytoplasmic HIV-1 RNA to translation and encapsidation. This might explain why Rev and tethered TAP exert the same pattern of pleiotropic effects.

Introduction

In contrast to simple retroviruses, HIV-1 as a lentivirus utilizes a number of trans-acting regulatory proteins which fulfill important functions throughout the different phases of the lentiviral replication cycle (recently reviewed in (16, 30, 34)). One of those regulators is the 16 kD HIV-1 Rev protein which is required for the expression of the structural proteins during the late phase of the HIV-1 replication cycle. This effect of Rev has been attributed to its nuclear export activity. After initial transcription of the integrated proviral genome “early” transcripts are subjected to the cellular splicing machinery. Alternative splicing events caused by the complex genome organisation of HIV-1 lead to expression of Rev from a multiply-spliced mRNA. Once Rev is translated in the cytoplasm it is imported into the nucleus where it binds to a viral RNA element, the Rev Response Element (RRE), which is present on
singly-spliced and unspliced transcripts. Rev seems to circumvent further splicing and leads
to an increase of the cytoplasmic levels of unspliced and singly-spliced viral transcripts by
tethering these transcripts to the Crm1 export pathway ((13, 28), reviewed in (36)). The
unspliced and singly-spliced transcripts serve as templates for translation and the unspliced
RNA is also encapsidated into assembling virus particles.

The magnitude by which Rev enhances lentiviral RNA levels in the cytoplasm has been
controversial. Under some experimental conditions, unspliced viral RNA levels in the
cytoplasm were only detectable in the presence of Rev while others observed just a 4-fold
enhancement of these cytoplasmic RNA levels by Rev (2, 8, 9, 12, 26, 36, 43, 45). Since Rev
was found to stimulate protein levels encoded by the Rev-dependent RNAs to a much larger
extent than the cytoplasmic levels of these RNAs, Rev also seems to stimulate translation ((6,
25, 35), recently reviewed in (17)). Consistently, Rev was furthermore shown to enhance the
association of the Rev-dependent viral RNA with polysomes (9).

Trying to develop RRE-deficient lentiviral vectors, we previously observed that the deletion
of the RRE led to a striking loss of infectivity despite nearly unchanged levels of unspliced
vector RNA in the cytoplasm of the producer cells (27). Further analyses revealed that Rev
only moderately enhanced unspliced vector RNA levels in the cytoplasm, but increased the
encapsidated RNA levels by two to three orders of magnitude (3). This effect was not due to
Rev-mediated stimulation of particle production, since a Rev-independent, codon-optimized
Gag-Pol expression plasmid lacking the RRE provided a constant excess of Gag (44).

The lentiviral vector RNA encapsidated in the experiments described above contained large
deletions of *gag-pol* and *env* also comprising cis-acting regulatory sequences (CRS) and
inhibitory sequences (INS) (8, 37–39). Since these sequences have been shown to modulate
Rev dependence, the minor effects of Rev on cytoplasmic lentiviral vector RNA levels could
simply be due to the absence of such regulatory sequences. Therefore, we have since
investigated the influence of Rev on almost full-length HIV-1 unspliced transcript levels in
Nuclear RNA Export and Packaging Functions of Rev

the cytoplasm and on the encapsidation process. Additionally, we have extended our analyses of the function of Rev to infected T cell lines and primary PBMCs.

Material and Methods

Plasmids

For the cloning of the proviral construct HIV_{Rev}^{4xMS2} the env ORF of pNL4-3Rev_{4xMS2} (47) was inactivated by the deletion of bases 7251-7254 (according to GenBank Accession Number AF324493). The rev ORF of the HIV_{Rev}^{4xMS2} construct was reactivated by site-directed mutagenesis leading to HIV_{Rev}^{4xMS2}. The expression plasmids encoding VSV-G (pHIT/G), HIV-1 Tat (pcTat), HIV-1 Rev (pcRev), a fusion of the coat protein of phage MS2 and human nuclear shuttling factor TAP (pMS2-hTAP) and codon-optimized gag-pol of HIV-1 (Hgp^{syn}) were kindly provided by M. Malim, J. Hauber, B. Cullen and R. Wagner, respectively, and have been described previously (7, 14, 29, 44).

Cell Culture

Cell cultures of HEK 293T and TZM-bl were maintained in DMEM (Gibco), 10 % FCS, Ciprofloxacin 20 μg/ml. Human T cell lines CEM-SS, HUT78 and Jurkat were cultured in RPMI1640 (Gibco), 10 % FCS, 20 μg/ml Ciprofloxacin. PBMCs were isolated from the blooduffy coat of three healthy donors using Ficoll400 centrifugation (400 x g, 30 min, 20 °C). PBMCs were cultured at 1x10^6 cells/ml in RPMI1640, 10 % FCS, 20 μg/ml Ciprofloxacin and activated by addition of 5 μg/ml PHA and 100 IU/ml IL-2 for 72 h.
Transfections

Transfections for the production of viral particles were done using the calcium phosphate coprecipitation method. Briefly, 1.5x10^6 293T cells were grown in 25 cm² flasks for 24 h and were transfected with 2 µg pcTat, 2 µg Hgp32, and with or without 2 µg pcRev or pMS2-hTAP, respectively. Unless stated otherwise, 500 ng of proviral construct per transfection were used.

0.1 µg pCMV-GLuc-1 (Targeting Systems, El Cajon, CA) expressing Gaussia Luciferase was added to each transfection to control for transfection efficiency. The medium was changed after 8 h to remove excessive plasmid DNA.

Transfections for Western blots were done using either calcium phosphate or PEI as described in (1). A PEI/DNA (v/w) ratio of 1.5 was used. The total amount of DNA transfected was adjusted to 10 µg per transfection by addition of calf thymus carrier DNA. Transfections were normalized using Gaussia Luciferase as an indicator for transfection efficiency and cell viability. The luciferase activities were used to adjust the RNA copy numbers of the RT-qPCRs for transfection efficiency.

RNA Isolation

For RNA isolation, cells were detached and washed in 1 ml PBS. The pellet was resuspended in 175 µl buffer RLN (50 mM TrisHCl, pH 8.0; 140 mM NaCl; 1.5 mM MgCl₂; 0.5 % Nonidet P-40 Substitute; 1000 U/ml RNase Inhibitor (Qiagen, Hilden, Germany); 1 mM DTT) and incubated for 5 min on ice. Debris and nuclei were pelleted (300 x g, 2 min, 4 °C) and the cytoplasmic fraction was transferred to 600 µl RLT buffer (Qiagen). For RNA isolation from cell nuclei, the nuclear pellet was washed in 500 µl PBS, again pelleted for 3 minutes at 300 x g, and then resuspended in 600 µl RLT buffer. The pellet was homogenized.
Nuclear RNA Export and Packaging Functions of Rev

using Qiashredder columns (Qiagen) at 13000 x g for 30 sec. The flow-through was
transferred twice onto the same column.

RNA isolation was done using the RNeasy Mini Kit (Qiagen) according to manufacturer´s
instructions followed by DNase digestion using the TurboDNA-free Kit (Ambion, Austin,
TX). The amount of total RNA extracted from the cytoplasmic and nuclear fraction was
determined by the Quant-iT RiboGreen RNA quantitation kit (Invitrogen, Karlsruhe,
Germany).

For RNA isolation from virus particles, supernatants of transfected cells were loaded on top
of a 30 % sucrose cushion and ultracentrifuged at 150000 x g for 2 h at 4 °C. Virus particles,
pelleted from 5 ml of cell culture supernatant were resuspended in 150 µl PBS. RNA was
isolated from the resuspended viral particles using QIAamp Viral RNA Mini Kit (Qiagen) and
eluted in 45 µl followed by DNase digestion using the TurboDNA-free Kit.

Quantitative RT-PCRs

HIV-1 unspliced RNA levels were determined using the QuantiTect Probe RT-PCR Kit
(Qiagen). Primer sequences homologous to a region of gag were taken from the Amplicor
HIV-1 Monitor test (31) and are specific for the unspliced HIV-1 RNA. Primers are pSK145
(AGT GGG GGG ACA TCA AGC AGC CAT GCA AAT) and pSKCC1B (TAC TAG TAG
TTC CTG CTA TGT CAC TTC C). Serial dilutions of an in vitro transcript were prepared as
a RNA standard with known copy numbers. The sensitivity of the assay was below 100
RNA copies/PCR, the inter-assay variability was 3 %. Cross reaction of the PCR with the
codon-optimized Hgp syn was not detected even after adding 10^8 DNA copies of Hgp syn. This is
consistent with the 7 to 8 mismatches between the primer sequences and their target sequence
of the codon-optimized gene. HIV-1 unspliced RNA copy numbers in 500 ng cytoplasmic or
nuclear RNA or 1 µl of the RNA extracted from the viral particles (corresponding to 6 – 26 ng
Nuclear RNA Export and Packaging Functions of Rev

p24) were determined in direct comparison to serial dilutions of the RNA standard. Elimination of transfected plasmid DNA was routinely confirmed by simultaneously analysing aliquots of one or two representative samples without adding the reverse transcriptase. Since non-encapsidated extracellular RNA is rather unstable we did not perform any additional RNase treatment.

Subcellular fractionation was also controlled with the QuantiTect Probe RT-PCR Kit (Qiagen) using primers preGAP-DHE6s (CCA CCA ACT GCT TAG CAC C) and preGAP-DHE6a (CTC CCC ACC TTG AAA GGA AAT) (4) homologous to the exon 6/intron 6 junction and the intron 6 of the unprocessed pre-mRNA of the glyceraldehyde 3-phosphate dehydrogenase gene, respectively. 500 ng of extracted nuclear and cytoplasmic RNA and serial dilutions of the nuclear RNA were subjected to the real-time RT-PCR. This allowed the calculation of the percentage of preGAPDH RNA in the cytoplasm relative to the nuclear preGAPDH RNA levels.

Protein analyses

For the analysis of the purity of cytoplasmic and nuclear fractions two flasks of 293T cells were transfected as described above. One half of the pooled cells was used to prepare total cell lysates by the addition of 500 µl of the stringent BLP lysis buffer (50 mM Tris-HCl (pH 7.4); 150 mM NaCl; 40 mM NaF; 5 mM EDTA; 5 mM EGTA; 1 % (v/v) Nonidet P-40; 0.1 % (w/v) Natriumdesoxycholat; 0.1 % (w/v) SDS). The other half was fractionated as described for the RNA analyses. Fractions were brought to a final volume of 500 µl using the BLP buffer.

Equal amounts of total protein were subjected to SDS-PAGE prior to the staining of the Western blots with antibodies to Histone H2B (Epitomics, Burlingame, CA), α-Tubulin (Rockland, Gilbertsville, PA), and GFP (Santa Cruz Biotechnology, Heidelberg, Germany).
Nuclear RNA Export and Packaging Functions of Rev

For the examination of Gag expression levels, transfected cells were lysed in 500 µl BLP. After adjusting to the same total protein content, lysates were separated by SDS-PAGE and p24CA was detected using antibody 183-H12-5C (NIH AIDS Research and Reference Reagent Program).

Infections

VSV-G pseudotyped viruses for the infectivity assay were prepared as follows: 293T cells were grown to 75% confluence in a 175 cm²-flask and transfected with 2 µg pcRev, 5 µg Hgp₃₃', 2 µg pcTat, 4 µg pHIT/G, and 10 µg of HIV₂⁻/₄xMS2 or HIV₂⁺/₄xMS2, respectively. 1.5 µg pEGFP-C1 and calf thymus carrier DNA to a total of 40 µg DNA were added to each transfection. Transfection medium was changed after 8 h. Supernatants were collected after 48 h and 72 h, filtered through a 0.45 µm filter, and stored at -80 °C in aliquots until use. Infectivity was measured on TZM-bl cells using β-galactosidase staining as follows: 5x10⁴ TZM-bl were seeded on a 24-well plate and grown for 24 h. Cells were treated with 200 µl serial dilutions of the virus containing supernatant for 4 h. The medium was changed and cells were grown for 2 days until fixation using 0.5% glutaraldehyde in PBS. After repeated washing in PBS, cells were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. For infection, T cells or PBMCs were pelleted and resuspended in the virus containing supernatant, thereby adjusting the MOI to 0.1. The medium was changed after 4 h. Cells were cultured for a further 36 h until RNA isolation.

Statistical analyses

To determine whether HIV-1 RNA levels in the cytoplasm and the particle differ significantly in the presence or absence of Rev or MS2-TAP, a two-sided T-test was used on the logarithmically transformed values for the RNA copy numbers.
Nuclear RNA Export and Packaging Functions of Rev

Results

To study the effect of Rev on unspliced HIV-1 RNA levels in the cytoplasm and genomic RNA encapsidation in the context of authentic viral sequences, HIV\textsuperscript{Rev}/4xMS2, a proviral construct with inactivating point mutations in \textit{rev}, was used (Fig. 1A). The construct also contained a 4 bp deletion in \textit{env} preventing virus replication. In addition, a part of the \textit{nef} open reading frame was replaced by four copies of a RNA stem loop of the bacteriophage MS2 to allow complementation of Rev by heterologous export factors (47).

The HIV\textsuperscript{Rev}/4xMS2 construct was transfected into 293T cells in the presence or absence of a Rev expression plasmid. A Tat expression plasmid was always included to enhance expression levels. Since Rev has a strong effect on Gag-Pol expression from proviral constructs, we provided excess levels of Gag-Pol in trans by cotransfection of Hgp\textsuperscript{syn}. The \textit{gag-pol} open reading frame of this plasmid has been optimized to mammalian codon usage without changing the amino acid sequence (44). Mutating approximately every fourth nucleotide of \textit{gag-pol} rendered the Gag-Pol expression completely independent of Rev. As expected, transfection of HIV\textsuperscript{Rev}/4xMS2 led to detectable levels of Gag expression only if cotransfected with the Rev expression plasmid (Fig. 1B). The addition of Hgp\textsuperscript{syn} led to a further increase of Gag expression levels which were clearly independent of the presence or absence of Rev (Fig. 1B). Thus, any effect of Rev on RNA encapsidation should not be due to the enhancement of Gag-Pol expression.

To determine an influence of Rev on the packaging efficiency of proviral RNA we performed a quantitative RT-PCR-based packaging assay. Briefly, 293T cells were co-transfected with HIV\textsuperscript{Rev}/4xMS2, a Tat expression plasmid and Hgp\textsuperscript{syn} in the presence or absence of a Rev expression plasmid. After 48 h, virus particles were pelleted through a 30% sucrose cushion and the particle-associated RNA was extracted. In parallel, cells were lysed under standard conditions to isolate cytoplasmic RNA. Quantitative RT-PCR was performed with primers targeting \textit{gag} in order to measure the amount of full-length HIV-1 RNA in the cytoplasm and...
Nuclear RNA Export and Packaging Functions of Rev

in the viral particles. Hgp30 encoded RNA is not detected in this PCR due to its extensive
codon optimization. As shown in Figure 2, Rev increased the cytoplasmic levels of the HIV-1
unspliced RNA after transfection of 1 µg HIV\(^{\text{Rev}/4xMS2}\) from \(8.9 \times 10^7\) to \(4.9 \times 10^8\) copies/µg
extracted RNA. In contrast to this modest increase, Rev enhanced packaged HIV-1 RNA copy
numbers approximately 4500-fold. To exclude a potential non-linear relationship between the
HIV-1 genomic RNA concentration in the cytoplasm and the packaging of this RNA into
virus particles, the amount of HIV\(^{\text{Rev}/4xMS2}\) DNA transfected was decreased in the presence
of Rev and increased in its absence. This led to experimental conditions under which the
cytoplasmic HIV-1 unspliced RNA concentrations were higher in the absence of Rev than in
its presence (Fig. 2). Even under these conditions, encapsidation was at least 350-fold higher
in the presence of Rev. The packaging defect in the absence of Rev was not due to inefficient
particle production, since the amount of p24 detected in the pelleted particles in the absence
of Rev was only 1.2 to 4.3-fold lower than with Rev (Fig. 2). This is consistent with previous
observations that the budding of the virus particles is independent of the amount of viral RNA
(15, 24). Thus, the major function of Rev seems to be the enhancement of packaging of the
genomic HIV-1 RNA, while there is only a modest effect on cytoplasmic levels of this HIV-1
RNA.

Only modest effects of Rev on cytoplasmic RNA levels of RRE-containing transcripts have
been observed before, particularly in the context of subgenomic constructs (9, 11, 26) but
have also been questioned due to concerns of contamination of cytoplasmic fractions with
nuclear RNAs (28). We therefore performed various experiments to determine the purity of
the cytoplasmic fractions. 293T cells were transfected as before including an EGFP
expression plasmid and the cytoplasmic fraction was recovered as described above. In
addition, the nuclear fraction was collected after a washing step of the nuclear pellet. Total
cell lysates as well as the nuclear and cytoplasmic fractions were then analyzed by Western
blot for EGFP, histone protein H2B and α-tubulin content (Fig. 3A). As expected, histone
H2B was detected in the nuclear fraction but not in the cytoplasmic fraction, while α-tubulin was present in the cytoplasmic fraction but not in the nuclear fraction. EGFP could be detected in both fractions confirming proper loading. Histone H2B could be more tightly associated with the nucleus than nuclear RNA and is therefore perhaps not a good marker for the contamination of the cytoplasmic fraction with nuclear RNA. We therefore determined the ratio of pre-mRNA levels of the endogenous Glyceraldehyde 3-phosphate dehydrogenase (preGAPDH) gene in the cytoplasm and the nucleus. RNA was extracted from the nuclear and cytoplasmic fractions of transfected 293T cells. The same amounts of extracted nuclear and cytoplasmic RNA and serial dilutions of the nuclear RNA were subjected to real-time RT-PCR with primers spanning intronic sequences of preGAPDH. Similar crossing points of the real-time RT-PCR results for the cytoplasmic RNA samples and a 1:30 dilution of the nuclear RNA preparation indicated that the concentration of the preGAPDH RNA in the cytoplasmic RNA was approximately 3% of the concentration of the nuclear RNA (Fig. 3B). Assuming an exclusive nuclear localization of the preGAPDH RNA, these results indicate that 3% of the extracted cytoplasmic RNA is actually derived from the nucleus. This nuclear contamination could only bias the results regarding the effect of Rev on the cytoplasmic RNA levels of the unspliced HIV-1 RNA, if the concentration of this RNA in the nucleus was substantially higher than in the cytoplasm. Therefore, we also quantified the HIV-1 full-length RNA copy numbers in RNA extracted from the nucleus and cytoplasm of HIV\textsuperscript{Rev}/4xMS2 DNA transfected cells. In the presence and absence of Rev the concentration of the unspliced transcript in the cytoplasmic RNA preparation was higher than in the nuclear preparation (Fig. 3C). This excludes the possibility that the full magnitude of the enhancing effect of Rev on the cytoplasmic levels of the full-length HIV-1 RNA is masked by a contamination with nuclear RNA.

Over-expression by transient transfection of epithelial 293T cells might lead to a spill-over of nuclear HIV-1 RNA into the cytoplasm in a Rev-independent manner. Therefore, we also
studied the effect of Rev on cytoplasmic RNA levels under rather natural expression levels in lymphoid cell lines which is considered to be a more relevant model for HIV infection. The CEM-SS, HUT78 and Jurkat lymphoid T cell lines were infected with VSV-G pseudotyped HIV particles transferring either Rev-deficient HIV\textsuperscript{Rev-}/4xMS2 genome or a Rev-proficient HIV\textsuperscript{Rev+}/4xMS2 genome. The \textit{rev} defect of the HIV\textsuperscript{Rev-}/4xMS2 construct was complemented \textit{in trans} during the production of the pseudotypes by co-transfection of a Rev expression plasmid. Vector titers were determined on TZM-bl cells in order to normalize the infectious dose to a multiplicity of infection of 0.1. Forty hours after infection cytoplasmic RNA was isolated as described above and the HIV-1 genomic RNA levels were compared between HIV\textsuperscript{Rev-}/4xMS2 and HIV\textsuperscript{Rev+}/4xMS2 infected T cell lines. Although all three cell lines were infected with the same stocks of pseudotypes, the effect of Rev on unspliced cytoplasmic HIV-1 RNA levels varied substantially (Fig. 4). In CEM-SS cells Rev enhanced the levels of the genomic transcript in the cytoplasm nearly 60-fold, while the 3- to 9-fold increase observed in Jurkat and HUT78 cells is similar to our observations in transfected 293T cells. Therefore, the HIV\textsuperscript{Rev-}/4xMS2 and HIV\textsuperscript{Rev+}/4xMS2 pseudotypes were also used to infect activated human peripheral blood monocytes (PBMCs) at a multiplicity of infection of 0.1. Determining the unspliced HIV-1 RNA levels in the cytoplasm of the infected PBMCs revealed that Rev increased these HIV-1 RNA levels just 3-fold (Fig. 4).

The nuclear export activity of Rev can be functionally replaced by constitutive transport elements (5, 18, 19, 33) or tethering of heterologous export factors to the HIV-1 RNA (46, 47). To determine whether this is also valid for the packaging effect of Rev the HIV\textsuperscript{Rev-}/4xMS2 DNA was co-transfected with an expression plasmid for a fusion protein consisting of the MS2 coat protein and the human Tip associated protein (hTAP), a cellular factor known to shuttle mRNA from the nucleus to the cytoplasm. Since the HIV\textsuperscript{Rev-}/4xMS2 RNA contains four copies of the MS2 stem loop in \textit{nef}, the MS2-hTAP fusion protein can bind to the HIV-1 RNAs. Similar to a previous study (46), the co-transfection of MS2-hTAP with HIV\textsuperscript{Rev-}...
Nuclear RNA Export and Packaging Functions of Rev

/4xMS2 DNA increased p24 levels approximately 140-fold (see Fig. 5B). However, co-
transfection of MS2-hTAP enhanced unspliced HIV-1 RNA levels in the cytoplasm only
marginally, while encapsidation of the HIV-1 genomic RNA into particles encoded by co-
transfected Hgp\textsuperscript{syn} was enhanced 280-fold (Fig. 5A). The tethering of hTAP to the MS2 stem
loops was important, since encapsidation of HIV\textsuperscript{Rev} genomic RNA lacking the MS2 stem
loops was strongly impaired even in the presence of MS2-hTAP. Although Rev enhanced
encapsulation to an even larger extent, the tethering of hTAP to the HIV-1 genomic RNA can
clearly rescue the packaging defect of a Rev-deficient HIV-1. To measure the effect of Rev
and MS2-hTAP on HIV-1 infectivity, VSV-G pseudotyped HIV\textsuperscript{Rev}/4xMS2 particles were
produced in the presence or absence of Rev or MS2-hTAP (Fig. 5B). Without Rev and MS2-
hTAP the infectious titer was below the detection limit. Adding Rev or MS2-hTAP increased
the infectious titer by at least 3000- and 500-fold, respectively. Since Rev and MS2-hTAP
also increased the secretion of Gag from HIV\textsuperscript{Rev}/4xMS2 transfected cells 533- and 140-fold,
respectively, the increase in infectivity could simply be due to enhanced particle production
rather than the enhancement of RNA encapsidation. Therefore, we again included Hgp\textsuperscript{syn} in
the co-transfection experiments. Adding Hgp\textsuperscript{syn} in the presence of Rev or MS2-hTAP did not
further increase the infectious titer (Fig. 5B). In the absence of MS2-hTAP and Rev, Hgp\textsuperscript{syn}
allowed the transfer of the HIV\textsuperscript{Rev}/4xMS2 construct, but the infectious titer was
approximately 100-fold lower than in the presence of Rev. Since transfection of Hgp\textsuperscript{syn} leads
to higher Gag expression levels than the co-transfection of HIV\textsuperscript{Rev}/4xMS2 and the Rev
expression plasmid, the infectivity correlates better with encapsidation efficacy than with Gag
expression levels.
Discussion

The observation that the tethering of TAP to the HIV-1 RNA enhances the encapsidation of the genomic RNA to a much larger extent than the cytoplasmic RNA levels indicates that the packaging function of Rev can be replaced by a heterologous protein. This is consistent with the recent observation that the replacement of the RRE by a constitutive transport element (CTE) leads to efficient genomic RNA encapsidation (32). Since the tethering of TAP to Rev-dependent RNA can also replace the nuclear export function of Rev (46), both activities of Rev seem to be mechanistically linked. However, this raises a number of questions regarding the precise mechanism by which Rev and TAP enhance the encapsidation process. How can both the Rev-mediated nuclear export via the CRM-1 pathway and the MS2-TAP triggered nuclear export render the cytoplasmic HIV-1 genomic RNA accessible to packaging, while the same HIV-1 genomic RNA reaching the cytoplasm in the absence of Rev or MS2-TAP is poorly encapsidated? This is particularly puzzling, since without a tethered export factor, the unspliced HIV-1 RNA is believed to reach the cytoplasm via the default TAP-mediated mRNA export pathway. One potential explanation could be that the rapid tethering of the unspliced HIV-1 RNA to either of the two nuclear export pathways prevents the formation of a ribonucleoprotein (RNP) complex that is incompetent for translation and encapsidation despite being exported to the cytoplasm. Evidence for the rapid interaction of Rev with the newly synthesized target RNA has been previously obtained (20). In addition to Rev and tethered TAP, CTEs were also shown to enhance the nuclear export and translation of Rev-dependent RNAs (18, 19, 33) as well as the infectivity of lentiviral vector particles (48). Since it is unlikely that different nuclear export factors and constitutive transport elements trigger the formation of RNP complexes mediating the same pattern of pleiotropic effects (enhancement of nuclear export, translation and packaging), the suppression of the formation of an inhibitory RNP complex by different RNA export factors and constitutive transport elements might be the more plausible mechanism. Whether any of the known cellular co-
Nuclear RNA Export and Packaging Functions of Rev

Factors of Rev (recently reviewed in (40)) is involved in the suppression of the formation of such an inhibitory RNP remains to be determined. The formation of a poorly accessible HIV-1 RNA protein complex in the absence of Rev might also explain an apparent discrepancy between full-length transcript levels in the cytoplasm as observed by in situ hybridisation and cellular fractionation experiments, respectively. In the absence of Rev there is hardly any cytoplasmic unspliced HIV-1 RNA detectable by in situ hybridisation (10, 23). In contrast, we and others have observed substantial levels of unspliced HIV-1 RNA in the cytoplasm by cellular fractionation experiments. Extensive control experiments for the fractionation argue against the assumption that high levels of full-length transcript detected in the cytoplasmic RNA preparation in the absence of Rev are due to the contamination with nuclear RNA. This clearly raises the possibility that the unspliced RNA present in the cytoplasm without Rev escapes efficient detection by in situ hybridisation. The mild denaturing conditions during in situ hybridisation might not be sufficient to resolve a tightly packed RNP complex that is formed in the absence but not the presence of Rev.

The magnitude of the effect of Rev on genomic HIV-1 RNA levels is not only influenced by the method used to measure the amount of HIV-1 RNA but also by the type of cells analyzed. In infected lymphoid cell lines, Rev enhanced cytoplasmic RNA levels from 3- to 60-fold. The latter value was obtained in CEM-SS cells. Interestingly, this cell line was also used in a previous study demonstrating that Rev is absolutely required for nuclear export (28). However, since Rev only enhances cytoplasmic RNA levels in primary PBMCs by a factor of 3, this strong dependence on Rev seems to be a particular property of CEM-SS cells.

Thus, the results of the present study challenge the general notion that the main function of Rev is simply to mediate nuclear export of viral RNAs. Interestingly, the nuclear export pathway of Gag encoding RNAs has also been observed to modulate the assembly of Gag proteins into particles (21, 22, 41, 42). Therefore, it rather seems that Rev modulates the RNP complex formed in the nucleus on RRE-containing viral RNAs, thereby either leading to a
Nuclear RNA Export and Packaging Functions of Rev

particular subcytoplasmic compartmentalization or affecting the accessibility of the unspliced HIV-1 RNA to translation and encapsidation.

The detection of substantial levels of unspliced HIV-1 RNA in the cytoplasm in the absence of Rev and therefore probably also during the early phase of the viral replication cycle could severely affect HIV-1 spread and persistence. If the structural proteins were indeed produced during the early phase of replication, infected cells could be lysed by antiviral effector mechanisms prior to the release and spread of progeny virus. Therefore, the Rev dependence of nuclear export and translation of the unspliced and singly-spliced HIV-1 RNAs might have actually evolved in order to increase the efficiency, by which expression of structural proteins during the early phase of the viral replication cycle is prevented. In the late phase of the replication cycle, Rev could then promote coordinated particle production and RNA encapsidation.

Acknowledgements

This work was supported through a grant from the German Research Foundation to K.Ü. (Ue45/11-1). B.G. is supported by the graduate course GRK 1045 funded by the German Research Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We are grateful to T. Grunwald, N. Ternette, V.V. Temchura and B. Tippler for experimental help and would like to thank M. Malim, J. Hauber, B. Cullen and R. Wagner for providing plasmids and D. Cosgrove for critical reading of the manuscript. TZM-bl cells were obtained from the EVA Centre for AIDS Reagents, NIBSC, UK and were donated by JC Kappes, X Wu, and Tranzyme Inc. The following reagent was obtained through the NIH AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious
Diseases: HIV-1 p24 Hybridoma (183-H12-5C), catalog number 1513 from Bruce Chesebro and Hardy Chen.

References


Nuclear RNA Export and Packaging Functions of Rev


Nuclear RNA Export and Packaging Functions of Rev


Nuclear RNA Export and Packaging Functions of Rev


Nuclear RNA Export and Packaging Functions of Rev


Nuclear RNA Export and Packaging Functions of Rev


Figure Legends

Figure 1: Rev-(in)dependent Gag-Pol expression.

A) Map of plasmids HIV$^{Rev/4xMS2}$ and Hgp$^{syn}$. The proviral HIV$^{Rev/4xMS2}$ construct contains the HIV-1 genome with inactivating point mutations in rev and env. In addition, parts of nef gene are replaced by 4 repeats of an RNA stem loop targeted by the MS2 coat protein (marked by the white box). Inactive ORFs are shown as black bars, active ORFs appear grey. The arrow indicates the region of the HIV-1 genome detected by the RT-qPCR.

Hgp$^{syn}$ encompasses a codon-optimized gag-pol ORF flanked by a strong heterologous promoter (CMV) and a polyadenylation (pA) signal. The codon-optimized ORF is hatched.

B) Western blot analysis with an HIV-1 p24 capsid antibody of total cell lysates transfected with proviral DNA (HIV$^{Rev/4xMS2}$) with or without the codon-optimized Gag-Pol expression construct (Hgp$^{syn}$) in the presence or absence of a Rev expression plasmid (upper panel). A GFP expression plasmid was also co-transfected to control for transfection efficiency by Western blot analysis with an anti-GFP antibody (lower panel).

Figure 2: Rev enhances packaging of HIV-1 genomic RNA.

Unspliced HIV-1 RNA copy numbers in the cytoplasmic RNA fraction and in the RNA extracted from viral particles are shown. The indicated amounts of HIV$^{Rev/4xMS2}$ DNA were co-transfected with a constant amount of Hgp$^{syn}$ in the presence or absence of Rev as indicated. Values shown are the means and the SEMs of 3 (*=2) experiments. The fold-enhancement of full-length HIV-1 RNA levels in the cytoplasm and the particles by Rev is given for the 1 µg dose of HIV$^{Rev/4xMS2}$. Statistical analysis of these results revealed that the difference between the HIV-1 RNA copy numbers is statistically significant (p < 0.01) in...
Figure 3: Purity of fractions.

A) Western blot analyses of different fractions of cell lysates. Cell lysates of transfected cells were normalized for total protein content and subjected to SDS-PAGE. Antibodies detecting histone H2B, α-tubulin, and GFP were used, respectively.

B) Cellular pre-mRNA of GAPDH was detected via RT-qPCR. The cytoplasmic RNA levels are given as percentage of nuclear RNA levels. Results shown are the mean +SEM of 4 experiments.

C) Unspliced HIV-1 RNA was detected in cytoplasmic and nuclear fractions using RT-qPCR. Samples were normalized for RNA content. The results shown are the mean +SEM of 3 experiments.

Figure 4: Effect of Rev on cytoplasmic RNA levels in infected lymphoid cells.

T cells were infected at an MOI of 0.1 with VSV-G pseudotyped HIV-1 constructs only differing in their Rev expression. Unspliced RNA copy numbers were determined in the cytoplasmic fractions by RT-qPCR. Shown are the means +SEM of 3 independent experiments. The fold-enhancement by Rev is given above the horizontal bars.

Figure 5: Functional replacement of Rev

A) Copy numbers of unspliced HIV-1 RNA in the cytoplasmic RNA fraction and in the RNA extracted from viral particles of cells co-transfected with HIV$^{Rev}$/4xMS2, HIV$^{Rev}$, a Rev
expression plasmid and/or the MS2-TAP expression plasmid. The mean values +SEM of 2-4 independent experiments are shown. The fold-enhancement of unspliced HIV-1 RNA levels in the cytoplasm and particles by MS2-hTAP is given. Statistical analysis revealed that the difference between the HIV-1 RNA copy numbers in the presence or absence of MS2-TAP is statistically significant (p < 0.05) in the particles, but not in the cytoplasm.

B) VSV-G pseudotyped HIV{}^{\text{Rev}/4xMS2} particles were prepared by cotransfection with or without Hgp{}^{\text{syn}} and expression plasmids for Rev or MS2-TAP. The mean values +SEM of the infectious titers of 2 to 3 independent experiments are shown. * Mean p24 concentration in the supernatant of cells co-transfected with HIV{}^{\text{Rev}/4xMS2} or cotransfected with HIV{}^{\text{Rev}/4xMS2} and Rev or MS2-TAP.