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# Regulation of HIV-1 *env* mRNA translation by Rev protein

Celia Perales[1,2], Luis Carrasco[1] and Maria Eugenia González[2*]

[1]Centro de Biología Molecular Severo Ochoa, Facultad de Ciencias, Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain

[2]Unidad de Expresión Viral, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera de Majadahonda-Pozuelo Km 2, 28220 Majadahonda, Madrid, Spain

*Corresponding author: Phone: 34 91 509 7072. Fax: 34 91 509 7919. e-mail: megonzalez@isciii.es

**Running title:** Enhanced *env* mRNA translation by Rev

**Key words:** HIV *rev*; HIV *env*; regulation of translation; Rev responsive element

**Abstract**

We have examined the effect of Rev on the regulation of the expression of RRE containing mRNAs when they were synthesised in the nucleus or directly in the cytoplasm. In the nuclear expression system, Rev enhanced *env* mRNA transport by about 1.6-fold, while translation of this mRNA was increased more than a 100fold. These findings indicate that the target of Rev activity is located mainly at the translational level. Synthesis of Env using a recombinant vaccinia virus system, that synthesised *env* mRNA directly in the cytoplasm, is also enhanced by Rev. Finally, RRE functioning was examined using a *luciferase* mRNA bearing this element. Rev stimulated the synthesis of Luciferase both when the *luc* mRNA was made in the nucleus or in cytoplasm. Our results indicate that the effect of Rev on *env* mRNA transport is low as compared with the enhancement of translation of this mRNA.

## 1. Introduction

Protein synthesis in human immunodeficiency virus type 1 (HIV-1) infected cells is regulated in a temporally manner by several factors including Rev protein (reviewed in [1]). Late viral genes express unspliced or single spliced mRNAs that require Rev protein to render Gag, Pol, Env, Vif, Vpr and Vpu. Several hypotheses have attempted to explain how Rev promotes the transport and translation of viral mRNAs containing the Rev responsive element (RRE). Most of these have emerged from studies focussing on the role played by Rev in aiding the transport of viral messengers from the nucleus to the cytoplasm, and/or on the increase in mRNA stability [2,3]. Rev interacts with the RRE that maps at conserved region of the HIV *env* gene [4,5]. A repressive sequence that overlaps the RRE domain is responsible for the low expression of viral structural genes by nuclear retention of unspliced or single spliced viral mRNAs [6]. The binding of Rev protein to the RRE motif induces efficient cytoplasmic export and translation of RRE-containing mRNAs [7]. Upon interaction with multiple cell cofactors, Rev promotes the translocation of Rev-RRE ribonucleoprotein particles across the nuclear membrane. The NES domain of Rev interacts with cellular CRM-1 protein, a member of the nucleoporin family [8]. This interaction has been suggested to facilitate the nuclear egress of HIV-1 transcripts bearing the RRE sequence [9]. Other sequences that differ from the RRE domain, the INS elements, may be responsible for the low expression of *gag/pol* genes, though the interaction of Rev with the RRE counteracts the negative effects of these inhibitory sequences [10].

As well as regulating the splicing and transport of RRE-containing mRNAs, Rev enhances their translation in the cytoplasm. Thus, Rev was able to stimulate protein synthesis in transfected 729 B cells by 27-fold, while only a 3-fold increase was observed in cytoplasmic intron-containing HIV RNA levels [11]. HeLa cells, constitutively expressing Tat, transfected with a *gag* expression plasmid, synthesised significant levels of *gag* mRNAs that localised in the cytoplasm, but their translation was low in the absence of Rev. Notably, Rev led to a 4 to 16-fold increase in cytoplasmic mRNA levels in HeLa cells and to an 800-fold rise in Gag protein synthesis [12]. *In vitro* translation of these mRNAs revealed their functionality even in the absence of Rev. HeLa cells transfected with a plasmidcontaining defective virus, in which the *pol*, *vif* and *vpr* genes are deleted, exhibited reduced viral mRNA levels in the cytoplasm in the absence of Rev. Moreover, the *gag* and *vpu*/*env* mRNAs present in the cytoplasm were not translated when the functional *rev* gene was lacking [13]. Cotransfection of a *rev* defective proviral mutant and a *rev* expression plasmid showed a Rev-dependent increase in the amount of *gag/pol* mRNA associated with polysomes. Similarly, the amount of *vpu/env* mRNA in polysomes was also increased [12]. Regulation of HIV-1 gene expression by Rev protein has been related to the targeting of RRE-containing RNA to components of the cellular translation complex, such as eIF5A in Xenopus oocytes [14] and lymphocytes [15]. Transfection of HeLa cells with a Rev-defective HIV expression plasmid gave rise to significant *gag* and *env* mRNA levels in the cytoplasm [16]. Under these conditions, efficient viral protein production was strictly dependent on the presence of Rev. Moreover,

association of *gag* mRNA with cytoskeletal proteins in the perinuclear clusters where viral protein synthesis

begins was only observed in the presence of Rev [16]. Here, we report that mRNA translation is enhanced by Rev, even when the *env* messenger is transcribed in the cytoplasm, indicating that nuclear transport is not necessary for the Rev induced stimulation.

## 2. Materials and methods

### *2.1. Constructs and antibodies*

To generate the expression vector pTM1-rev, the coding region of HXB3 Rev [17] was amplified by polymerase chain reaction from pCMVinsrev plasmid, kindly provided by B.R. Cullen (Duke University. Durham, NC. USA). The PCR amplified cDNA fragment was inserted into the *Nco* I and *Bam*H I sites of pTM1.

The primers used were: 5' (GGGGCCCATGGCAGGAAGAAGCGGA), and 3' (GGGCCCAGATCTCTATTCTTTAGCTCC). The pCDNA-rev construct was

obtained by inserting the *Kpn* I/*Xho* I *rev* fragment from pTM1-rev into the pCDNA plasmid. The pKS-env vector was constructed from pHXB2-env plasmid [18], obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The *Eco*R I/*Bcl* I fragment obtained by enzyme digestion was inserted into the pBluescript KS. Similarly, *Eco*R I/*Bcl* I fragment encoding *env* gene was inserted into pTM1 plasmid. The pCDNA-env plasmid was constructed by cloning the *Eco*R I/*Xho* I fragment of pTM1-env into the pCDNA plasmid. pKSluc contains a *Bam*H I fragment with intronless sequence of the firefly *luciferase* gene [19]. Downstream from the reporter gene, a PCR fragment containing 351 nucleotides of RRE region [20] (7066-7416 fragment of isolate BH10) was inserted in the pKS-luc plasmid to generate pKS-RREluc. The primers used were 5'

(GGGGGGGATATCTAGCACCCAAGG) and 3'

(GGGGGGAAGCTTTAGCATTCCAAGGCA). The PCR fragment was digested

with *Eco*R V and *Hind* III and then inserted into pKS-luc plasmid, which previously was digested with *Sma* I and *Hind* III enzymes. pCDNA-luc and pCDNA-RREluc were directly obtained from pKSluc and pKS-RREluc by restriction digestion, using *Apa* I/*Not* I and *Bam*H I/*Xho* I, and insertion of the luc and RREluc fragments, respectively, into the pCDNA plasmid. The monoclonal antibody to HIV-1 Rev, generously provided by Dr J. Karn (Cambridge, UK.), and the rabbit serum antiHIV-1/gp120, donated by Dr S. Ranjbar (Herts, UK.), were obtained from the NIBSC Centralised Facility for AIDS Reagents (supported by the EU Program EVA and the UK Medical Research Council).

### 2.2. Transfection and luciferase assays

The *env* and *rev* genes were efficiently expressed, under the control of the bacteriophage T7 promoter, in human HeLa and simian COS cells infected with T7-recombinant vaccinia virus and transfected with the pKS-env and pTM1-rev plasmids, respectively, using lipofectin reagent (Gibco-BRL) [21]. Both genes were also expressed from pCDNA-env and pCDNA-rev plasmids transfected in human 293 cells using a calcium phosphate protocol [22]. Luciferase constructs of pCDNA-luc and pKS-luc were also transfected using calcium phosphate or lipofectin reagent, respectively. Luciferase activity was measured as described

[23].

### 2.3. Western blotting

Entire cell lysates were fractionated on sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) gels with 10% (anti-gp120) or 15% (anti-Rev blots) polyacrylamide, transferred to nitrocellulose membranes by wet immunotransfer, and processed for Western blotting. The blots were probed with an antibody to gp120 or Rev. Goat-peroxidase-conjugated anti-rabbit or antimouse antibodies (Pierce) and the ECL kit (Amersham) were used to detect bound antibodies. Chemiluminescence was detected by exposure to Agfa X-ray film.

### 2.4. Cell fractionation in nucleus and cytoplasm

Forty eight hours after transfection, the 293T cell monolayer was washed with PBS and the cells then harvested in buffer containing 10 mM Tris-HCl [pH

8.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mg/ml yeast tRNA, 2 mM RNAse inhibitor and 0.5% IGEPAL CA-630. After mixing, the cell extract was incubated at 4 ºC for 5 min and then centrifuged at 3,000 rpm and 4 ºC for 2 min. The supernatant and pellet fractions were used to analyse protein and RNA contents in the cytoplasm and nucleus of transfected cells.

### 2.5. Reverse transcription and PCRs

Total RNA was purified from the cytoplasmic fraction of transfected cells using the TRIzol reagent (Invitrogen), DNAse I treated and phenol-chloroform extracted. *env* mRNA was amplified using the RT-PCR one step protocol in a total volume of 50 µl. The reaction mixture containing: 0.5 µg RNA, 10 µl reaction buffer

(Promega), 10 mM of each dNTP, 50 pmoles of each primer (5'

GGGGGGGATATCTAGCACCCACCAAGG and 3'

GGGGGGAAGCTTTAGCATTCCAAGGCA), 25mM $MgSO_4$, and 5 units of AMV and *Tfl* polymerases was incubated at 48 ºC for 45 min for the reverse transcription reaction. Amplification reactions were performed for 30 cycles at 94 ºC for 30 sec, 60 ºC for 1 min, and 68 ºC for 2 min, with a final extension at 68 ºC for 7 min. Real time PCR reaction was

carried out using the Light Cycler-FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions. Briefly, the cDNA obtained by reverse transcription was amplified in 10 µl of reaction volume, containing 1.2 µl reaction mix, 3.5 mM $MgCl_2$ and 10 µM of each primer. The amplification reaction was performed at 95 ºC for 10 min, followed by 45 cycles at 95 ºC for 5 sec, 59 ºC for 5 sec and 72 ºC for 10 sec. To determine mRNA concentration, a control RT-PCR quantitative reaction was performed using standard cell oligonucleotides in which glyceraldehyde 3-phosphate dehydrogenase was amplified.

### *2.6. Immunoprecipitation*

Cells were metabolically labelled with a [$^{35}$S]methionine-cysteine mixture (*Amersham*) (1.2 mCi/ml) for 1 hour. The cells were then washed with ice-cold

PBS, harvested in immunoprecipitation buffer (250 mM NaCl, 5 mM EDTA, 25 mM Tris-HCl (pH 7.5) and 1% NP-40) and incubated at 4 ºC for 30 min. The cell lysate was centrifuged at 10,000 x g for 15 min to remove particulate debris. The supernatant was incubated for 1 h at 4 ºC with protein A-Sepharoseimmunoglobulin G complexes and then centrifuged at 10,000 xg for 20 sec. The precleared supernatant was incubated overnight with specific antibody. Immune complexes were precipitated with protein A-Sepharose for 1 h at 4 ºC. After three washes with immunoprecipitation buffer, analysis of precipitate complexes was carried out by SDS-PAGE and autoradiography.

## 3. Results

### *3.1. Stimulation of Env synthesis by Rev using a nuclear expression system.*

Initially, our aim was to assess whether Rev could stimulate the expression of *env* obtained from an HXB2 HIV-1. To this end, the cDNA encoding Env, together with its 5' leader sequence, was introduced in a pCDNA plasmid, an expression vector containing the CMV promoter. The Rev encoding sequence of HIV-1 was also introduced in a pCDNA plasmid. Both recombinant plasmids were then used to cotransfect human 293 cells. Immunofluorescence analysis with nonpermeabilized cells revealed the location of HIV-1 glycoproteins at the cell surface only when Rev was present (results not shown). Our next goal was to estimate the amount of cytoplasmic *env* mRNA occurring in the absence or presence of Rev. This was achieved by lysing and separating cotransfected cells 48 h.p.t. into nuclear and cytoplasmic fractions. The amount of HIV-1 Env in the cytoplasmic fraction was then determined by western blotting. Figure 1A shows that gp160 is only observed when Rev is expressed. This demonstrates that in this system and under the conditions used there is a profound stimulation of Env synthesis only in the presence of Rev. The extent of Rev stimulation of Env synthesis could not be determined by densitometric scan analysis since no gp160 was detected in the absence of Rev. However, bearing in mind the sensitivity of this assay, this quantification suggested that it was several hundred fold. This finding agrees well with the enhancement noted using other systems [12]. As a control, luciferase synthesis was also determined (Figure 1B). Almost no differences were found in cytoplasmic luciferase

in the absence or presence of Rev. This finding indicates that Rev does not unspecifically enhance the synthesis of any protein. Besides, the nuclear fraction showed no luciferase activity (data not shown), indicating no contamination from the cytoplasm. This can also be inferred upon analysis of the RNAs present in both fractions (Figure 1C). To estimate the amount of *env* mRNA, RT-PCR analysis of total purified RNA, after DNAse I digestion, was carried out. This PCR amplification yielded a RRE fragment from *env* mRNA (Figure 1D). The *env* amplified product was quantified by real-time PCR. Rev stimulated the amount of *env* mRNA present in the cytoplasm 1.6-fold (Figure 1E). Our results indicate that although Rev enhances the amount of cytoplasmic *env* mRNA, most of the enhanced Env synthesis observed in this system occurs directly at the translational

level.

### *3.2. Stimulation of Env synthesis by Rev using a cytoplasmic expression system*

Once we had established that nuclear synthesised *env* mRNA responded to Rev, a cytoplasmic expression system was used to explore Env synthesis in the presence or absence of Rev. To this end, the vaccinia recombinant virus (VT7) and a T7 promoter driven plasmid (pTM1) were used to simultaneously express Rev and Env proteins. Cotransfection of both plasmids was performed in HeLa or COS cells. Initially, different pTM1-rev concentrations were assayed keeping constant the amount of pKS-env. Figure 2 (Panel A) shows the western blotting using antibodies against Env. Clearly, the accumulation of Env is dependent on the amount of pTM1-rev employed as estimated by western blotting. This stimulation was more apparent in HeLa cells than in COS cells. Higher concentrations of this plasmids interfered with the transfection assay (Data not shown). In order to test the actual synthesis of Env, transfected cells were radioactively labelled and immunoprecipitation was carried out. An enhancement of Env synthesis of about 6-fold was noted in HeLa cells in the presence of Rev, while no such enhancement was observed in COS cells (Figure 2B). This suggests that Rev is able to stimulate the translation of the *env* mRNA present in the cytoplasm of human cells, but not in those of simian origin. Alternatively, it could be that Rev does not stimulate Env synthesis in COS cells, because mRNA translation is sufficiently efficient in the absence of Rev. As a control, Rev synthesis was determined when the pTM1-rev was employed in both cell types; a higher expression level was observed using COS cells (Figure 2C). These findings are in accordance with previous results illustrating that gene expression using the VT7 system is more efficient in COS than in HeLa cells [24].

The aminoglycoside antibiotic neomycin B blocks the stimulation of RRE- containing mRNAs by Rev [25]. The presence of this compound inhibited the stimulation of Env synthesis by Rev in the cytoplasmic system using recombinant VT7 in HeLa cells (Figure 2D). The antibiotic failed to block total protein synthesis, indicating that the inhibition of Env synthesis stimulation is selective. We can therefore conclude that the enhancement of Env synthesis by Rev in the HeLa cell cytoplasmic system is mediated by the Rev/RRE interaction. These findings further support the idea that Rev is able to stimulate the translation of an mRNA synthesised in the cytoplasm.

### *3.3. Translation of luciferase mRNA containing RRE*

To compare Rev activation of the translation of RRE-containing mRNAs with that of mRNAs lacking this element, the two expression systems described above were employed. Two different luciferase encoding mRNAs were tested, one of which bears the RRE element (351 bp) [20] as depicted in Figure 3A. The responsiveness of these mRNAs to Rev when they are synthesised in the nucleus was analysed by measuring luciferase activity at 48 h.p.t. (Figure 3B). Almost no differences in luciferase activity were noted in the absence or presence of Rev in conditions of two different *rev*/*luc* plasmid proportions when the RRE-lacking *luc* mRNA was tested. The two *rev*/RRE*luc* plasmid proportions analysed gave rise to a 2- or 3-fold stimulation of luciferase synthesis. Thus, although the presence of the RRE in the *luc* mRNA confers some stimulation capacity upon Rev, this enhancement seems to be far below that related to the more physiological mRNA, *env* mRNA. Notably, it was also possible to observe clear stimulation of luciferase synthesis by Rev when the VT7 system was employed (Figure 3C). This stimulation may reach 3-fold. This level of Rev-induced activation of RRE*luc* mRNA translation was similar to that observed using the nuclear system. Moreover, this luciferase activity enhancement was dependent on the presence of RRE in the

mRNA.

## 4. Discussion

Gene expression in HIV-infected cells is regulated at both the transcriptional and translational level by a number of cellular and viral factors. The regulatory functions of Rev are exerted at several posttranscriptional steps, i.e., the modulation of splicing, transport of mRNAs from the nucleus to the cytoplasm and enhanced translation of RRE-containing mRNAs. Upon transcription and processing, mRNAs interact with several proteins that govern their transport through nuclear membrane pores. The interaction of Rev with an array of these proteins promotes the exit of viral mRNAs that posses the RRE motif from the nucleus. The dependence of this transport on Rev may vary from one cell type to another. Thus, in some cells, the cytoplasmic accumulation of RRE-containing viral mRNAs is not much affected by the presence or absence of Rev. However, in other cells, the transit of these RNAs through the nuclear membrane is largely dependent on Rev function. In the human 293 cell line used here, Rev moderately enhanced the transport of *env* mRNA to the cytoplasm. This suggests that Rev function is replaced, at least in part, by a cell protein or that Rev fails to interact with these nucleoprotein components at the nucleus of 293 cells.

Several investigations have shown that Rev increases the translation of different mRNAs bearing the RRE [12,26,27]. Complexed with several cell proteins, these mRNAs can efficiently enter polysomes in the presence of Rev. Unless Rev is functional, the simple

presence of these mRNAs in the cytoplasm is insufficient for their recognition by the translational apparatus. In the present set of experiments, we found that the translation of an HIV mRNA encoding Env which is transcribed in the nucleus of 293 cells is largely dependent on Rev. Thus, in these cells the translational apparatus does not recognise the *env* mRNA in the absence of Rev. Perhaps the binding of Rev to RRE acts as a signal identifying which mRNAs should be recognised by the translational machinery. Interaction of Rev with the components involved in the initiation of translation, including eIF5A, could be a prerequisite for these mRNAs to enter the polysomes. Alternatively, Rev may bind to other cell factors which inhibit mRNA translation to derepress the synthesis of Env.

Our findings certainly reveal that Rev enhances Env synthesis when its messenger is synthesised in the cytoplasm. In the vaccinia-VT7 system used here, there is no transport of mRNAs through the nuclear pores, yet Env synthesis is still enhanced by Rev. As to the role played by RRE, it seems clear that the simple presence of this motif on the *luc* mRNA does not confer Rev its stimulating capacity. However, the extent of stimulation is far below that found when a more physiological mRNA, such as the *env* mRNA, is used in the nuclear system. This means that besides RRE, there is a need for other elements or it is perhaps the global architecture of the HIV mRNA that is responsible for the regulatory role of Rev.

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## Figure legends

**Fig. 1. Effects of Rev on RRE *env* mRNA and Env protein in the cytoplasm of**

**293 cells.** 293 cells were transfected with 0.25 µg control pCDNA or pCDNA-env plasmids in the presence (+) or absence (-) of 0.5 µg pCDNA-rev. Forty-eight hours posttransfection, the cells were harvested and nuclear (N) and cytoplasmic (C) fractions separated. *Panel A*: Cytoplasmic fraction was analysed by Western blotting with Env polyclonal antiserum. *Panel B*: Luciferase activity was determined 48 h.p.t. in the cytoplasmic fraction of 293 cells. Transfections were performed using 0.25 µg pCDNA-RREluc with/without 0.5 µg pCDNA-rev. *Panel C:* Ethidium bromide staining after electrophoresis of agarose gel loaded with total RNA from cells transfected with pCDNA-env and pCDNA-rev. *Panel D*: RT-PCR amplification of cytoplasmic RNA using oligonucleotides detecting the cDNA of Env. Lane –RT corresponds control PCR without RT from cells transfected with pCDNA-env and lane C corresponds control RNA obtained by *in vitro* transcription from pKS-env plasmid. *Panel E*: Quantifying cytoplasmic *env* mRNA by real-time polymerase chain reaction.

**Fig. 2. Effect of Rev on Env synthesis using the recombinant vaccinia VT7 system.** HeLa and COS cell monolayers, treated with 40 µg /ml of ara-C, were infected with T7 recombinant vaccinia virus and subsequently transfected with 0.25 µg pKS or pKS-env in the presence (+) or absence (-) of 0.25 or 0.5 µg pTM1-rev plasmid. Sixteen hours posttransfection gp160 accumulated into cells was detected by western blotting using Env antiserum (*Panel A*). Similar infection/transfection experiment was performed using optimal concentration of pTM1 rev plasmid (0,5 µg). Fifteen hours posttransfection, the cells were pulse labelled with [$^{35}$S]methionine for 1 hour and analysed by immunoprecipitation using Env polyclonal antiserum (*Panel B*). The bar chart (below) shows the quantification of the Env protein band *Panel C:* Western blotting using the Rev monoclonal antibody. *Panel D:* Two hours posttransfection, HeLa cells were treated (+) or not (-

) with 10 mM neomycin B. Thirteen hours later, the cells were pulse labelled with [$^{35}$S] methionine for 1 hour and immunoprecipitated with Env antiserum. The samples were analysed on polyacrylamide gels and visualised by autoradiography before (right) and after (left) immunoprecipitation.

**Fig. 3. Effect of Rev on luciferase synthesis from RRE-containing plasmids.** *Panel A*: Schematic representation of the reporter plasmids. *Panel B*: Expression of the *luciferase* gene in a nuclear expression system. 293 cells were cotransfected with 0.5 µg control pCDNA (empty bars) or pCDNA-rev (black bars) and 0.1 µg (left graph) or 0.05 µg (right graph) pCDNA-luc or pCDNA-RREluc plasmids. Forty eight hours after transfection, luciferase activity was determined. *Panel C*: Expression of the luciferase gene in a cytoplasmic expression system. HeLa cells treated with 40 µg /ml ara-C were infected with T7 recombinant vaccinia virus and subsequently cotransfected with 0.5 µg control pTM1

(empty bars) or pTM1-rev plasmids (black bars) and 0.1 µg (left graph) or 0.05 µg (right graph) pKS-luc or pKS-RREluc.

Sixteen hours posttransfection, luciferase activity was determined.