Rev: beyond nuclear export

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Rev remains a hot topic. In this review, we revisit the insights that have been gained into the control of gene expression by the retroviral protein Rev and speculate on where current research is leading. We outline what is known about the role of Rev in translation and encapsidation and how these are linked to its more traditional role of nuclear export, underlining the multifaceted nature of this small viral protein. We discuss what more is to be learned in these fields and why continuing research on these 116 amino acids and understanding their function is still important in devising methods to combat AIDS.

Introduction

The year 2008 marked the 25th year of human immunodeficiency virus (HIV) research. Such a milestone often prompts an assessment of what has been accomplished and where gaps remain in our knowledge. As with most things in their mid-twenties, HIV research has achieved a great deal, but there is still a lot to learn. This dichotomy is exemplified by the viral regulatory protein Rev. Most reviews specifically addressing Rev function date from the 1990s; this is surprising, given the diversity of roles attributed to it since then. Rev is best known to stimulate nucleocytoplasmic transport of incompletely spliced viral RNAs (Cullen, 1998a, b; Hope, 1999; Pollard & Malim, 1998) within the HIV-1 life cycle (Fig. 1). Rev binds to RNAs containing the Rev-response element (RRE), which is contained within all incompletely spliced RNAs (Fig. 2a, b). The domain organization of Rev is shown in Fig. 2(c). The amino-terminal domain of Rev contains the RNA-binding activity of the protein (Daly et al., 1989; Green & Zapp, 1989; Hope et al., 1990; Southgate et al., 1990; Zamore et al., 1990; Zapp & Green, 1989) and is an arginine-rich sequence that also serves as the nuclear-localization signal (Berger et al., 1991; Bohnlein et al., 1991; Kubota et al., 1989; Malim et al., 1989b; Perkins et al., 1989). Regions surrounding the amino-terminal domain are proposed to be important in the multimerization of the protein onto the RNA (Bogerd & Greene, 1993; Madore et al., 1994; Olsen et al., 1990; Zapp et al., 1991). The carboxy-terminal domain, originally referred to as the activation domain (Malim et al., 1991; Venkatesh & Chinnadurai, 1990; Weichselbraun et al., 1992), contains the nuclear-export signal (Fischer et al., 1995; Wen et al., 1995). The combined effects of the nuclear-localization and nuclear-export signals allow Rev to shuttle in and out of the nucleus via interactions with importin-β and CRM-1, respectively (see inset in Fig. 1). This allows incompletely spliced RNAs access to the translation machinery and makes full-length genomic RNA available for encapsidation into virus particles.

The focus of Rev research has leaned traditionally towards control of splicing (Chang & Sharp, 1989) and transport. However, like most things retroviral, the Rev story turns out to be more complex than this. Rev is reported to play a role in a variety of aspects of the retrovirus life cycle, including polyadenylation (Barksdale & Baker, 1995; Campbell et al., 1994), RNA stability (Malim & Cullen, 1993), translation and encapsidation. These functions have often proven difficult to separate in experimental systems. The latter two are the main subjects of this review.

Rev and translation – early hints

Rev plays an important role in the nucleocytoplasmic export of RNAs containing the RRE (outlined schematically in Fig. 1; Cullen, 2003; Pollard & Malim, 1998). Interestingly, the earliest research on Rev found that mutagenesis of sequences that we now know as Rev did not alter the ratio of nuclear to cytoplasmic viral RNA, despite a severe defect in viral protein production (Sodroski et al., 1986). Tat and Rev also caused a dramatic increase in reporter-protein expression, disproportionate to the moderate effect on RNA levels (Knight et al., 1987). Similarly, while investigating the nature of the effect of Rev on transport of viral RNA species throughout the cell, Emerman et al. (1989) observed a 2-fold increase in Env-specific RNA, but this was accompanied by a 50-fold change in protein level, a finding echoed later the same year by Hadzopoulou-Cladaras et al. (1989). Interestingly, this discrepancy has also been observed for human T-cell leukemia virus 2 (HTLV-2) Rex (Kusuhara et al., 1999). This consistently observed disparity foreshadowed later investigation of a potential role of Rev in enhancing viral translation.
Translation as a critical control point for gene expression in HIV

HIV-1 encodes nine proteins on a single 9.2 kb genomic RNA and must therefore employ a number of strategies, including alternative splicing, polyprotein processing, leaky scanning, frameshifting and reinitiation, to produce these proteins (Bolinger & Boris-Lawrie, 2009). All RNAs from which these proteins are transcribed contain the same complex 5′ structure, including the transactivation-
response element, poly(A), primer-binding site and packaging signal stem–loops. These structural motifs inhibit translation initiation and ribosomal scanning (Geballe & Gray, 1992; Miele et al., 1996; Parkin et al., 1988), making it important for the virus to counteract this by both stimulating translation from its own RNAs and compensating for its reported ability to cleave host translation factors (Alvarez et al., 2003, 2006; Collier & Gray, 2006). A wide range of mechanisms have been developed by viruses to increase viral protein expression, often at the expense of cellular translation and commonly acting through translation-factor cleavage or phosphorylation (Mohr et al., 2007). However, HIV relies on cellular viability and should ideally enhance its own translation without impairing that of cellular mRNAs.

**Rev and translation – accumulating evidence**

Arrigo & Chen (1991), investigating the effect of Rev on cytoplasmic accumulation of viral RNAs and protein expression in COS and lymphoid cells, noted, as before, that the level of gag RNA in the cytoplasm of COS cells was reduced in the absence of Rev. A slight reduction was seen in the level of singly spliced RNA species in the cytoplasm of cells transfected with wild-type Rev, rather than with the Rev-deleted construct. However, in 729 B cells, no such

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**Fig. 2.** (a) Schematic of the HIV-1 genome, showing open reading frames as shaded rectangles and the LTRs as open rectangles. (b) Schematic of the full-length genomic RNA with the positions and structures of the Rev-binding sites (loop A in stem–loop I of the packaging signal and the RRE) indicated below. The RRE structure is taken from Pollard & Malim (1998). (c) Domain organization of the HIV-1 Rev protein. NES, Nuclear-export signal; NLS, nuclear-localization signal.
disparity was observed. On electroporation with wild-type or Rev− plasmid DNA constructs, cells contained no detectable Env or Gag in the absence of Rev expression. This reduction in the amount of Gag protein was more marked than could be explained by the reduced RNA level. These researchers went on to investigate the polysomal association of cytoplasmic RNAs in cells transfected with wild-type and Rev− constructs as a measure of translational activity of those RNAs (Lewin, 1997). The spliced RNAs tat and rev showed similar polysomal fractionation profiles on sucrose gradients for both wild-type and mutant constructs, but association of unspliced env/vpu RNAs with polysomes was reduced significantly in the absence of Rev.

The same group then hypothesized that Rev would continue to associate with RRE-containing RNAs in the cytoplasm post-nuclear export (Arrigo et al., 1992). Rev–RNA binding was analysed in cytoplasmic extracts of phytohaemagglutinin-stimulated peripheral blood lymphocytes. gag/pol, vif and vpr RNAs associated strongly with Rev in the cytoplasm, whereas tat/rev RNAs did not associate at all, implying that the RRE was responsible for this interaction; however, env/vpu RNA showed an intermediate phenotype. There was little or no specific interaction between Rev and any RNA in cells transfected with RRE− or Rev− proviral clones. As expected, these mutants showed reduced levels of incompletely spliced RNAs and increased levels of fully spliced RNAs. Rev was associated with env/vpu and vif RNAs more in the cytoplasm than in the nucleus. A Rev mutant, wild-type for all functions except nuclear export, had impaired protein production and nuclear-export function, as described above. This mutant, in contrast to the wild-type Rev, was able to interact specifically with viral RNAs in the nucleus as well as the cytoplasm, suggesting that Rev was required for nuclear export of RRE-containing RNAs and that continued association of Rev with these RNAs in the cytoplasm facilitated interaction with the translation machinery.

Lawrence et al. (1991) examined the intracellular distribution and translation of env mRNA by using two different Rev-dependent expression systems. Using a pSVAR expression system where tat and env sequences were located downstream of a chloramphenicol acetyltransferase (CAT) reporter, significant levels of mRNA were seen in the cytoplasm and nucleus in both the presence and absence of Rev. A significant enhancement of CAT activity was observed with no significant change in RNA distribution, leading the authors to conclude that expression of Rev facilitates utilization of cytosolic mRNA by the cellular translation apparatus. In a splicing-dependent reporter system, with authentic splice sites, this was not seen. The difference between these two systems may reflect the sequence context and/or interactions with different cellular factors. SVAR RNAs might associate with ribonucleoprotein (RNP) complexes that specifically increase the efficiency of translation from those RNAs. These data also suggest that the nuclear Rev–RRE interaction may not be a prerequisite for subsequent translation stimulation.

Using a plasmid producing only unspliced gag RNA and consisting of the 5′ long terminal repeat (LTR), the gag gene and the RRE linked to the polyadenylation signal of simian virus 40, D’Agostino et al. (1992) also addressed the association of HIV-1 RNAs with polysomes. Transfection into HeLa cells that express Tat constitutively again revealed a large discrepancy between cytoplasmic gag mRNA and Gag protein levels. Rev increased RNA and protein levels by 4.4- and 845-fold, respectively. gag mRNA produced in the presence of Rev was detected in the polysomal fractions, whereas that produced in the absence of Rev was not. By using a Gag expression plasmid lacking the RRE, polysomal association was deficient in both the absence and the presence of Rev, again invoking a Rev–RRE interaction as being essential for gag RNA association with polysomes. This was confirmed by Northern blot analysis of Tat-expressing HeLa cells transfected with the gag–RRE plasmid in the absence and presence of Rev. In the absence of Rev, gag RNAs localized to the perinuclear region rather than the cytoplasm. Subsequent publications have reproduced this work and suggest that Rev–RNA clusters may colocalize with β-actin (Kimura et al., 1996).

Further support for a translational role for Rev came from Perales et al. (2005), who addressed the discrepancy between the fold increase in unspliced RNA and its cognate protein in the cytoplasm in the presence and absence of Rev. A 1.6-fold increase in RNA was observed, compared with a 100-fold increase in protein; this is consistent with, if less marked than, previously published data. By using a recombinant vaccinia virus system that synthesizes env mRNA directly in the cytoplasm, Env levels were also enhanced by Rev. In experiments using T7 recombinant vaccinia virus, Rev expression plasmids and plasmids encoding RRE+ or RRE− luciferase reporter RNAs to exclude nucleocytoplasmic-transport effects, the RRE-containing construct was also stimulated by Rev.

Much evidence therefore points towards Rev having a positive effect on translation of incompletely spliced HIV-1 RNAs in virus-infected cells, but there are caveats, including the variable effects seen with differing systems and cell types and the risk that a system may generate artefactual effects because it is overloaded with RNA.

A second Rev-binding site

The RRE seems not to be the only Rev-binding site in the HIV-1 genomic RNA. Initially identified as having structural homology to the RRE (Greatorex et al., 2002), a second binding site in the 5′ untranslated region (UTR) of the HIV-1 RNA was confirmed to bind Rev specifically by electrophoretic mobility-shift assay, surface plasmon resonance and nuclear magnetic resonance. The site is on stem–loop I of the packaging signal and is referred to as loop A (Gallego et al., 2003; Fig. 2b). It consists of AGGA
Motifs in the 5’ end of a retroviral RNA enhancing expression of downstream open reading frames are not new (reviewed by Boris-Lawrie et al., 2001). The spleen necrosis virus (SNV) LTR is able to facilitate Rev–RRE-independent HIV-1 Gag expression (Butsch et al., 1999) through sequences within the LTR, which are position-dependent. The RU5 and U3 regions possibly act synergistically to produce this effect, mediating a 2- to 4-fold increase in cytoplasmic accumulation of viral RNAs, insufficient to account for the increase seen in Gag expression. The SNV LTR increased polysomal association of spliced and unspliced viral RNAs by 2- to 3-fold, but again, this was insufficient to account for the transactivation of Gag expression, making a case for an increase in translational efficiency mediated by LTR sequences. Stimulation of translation by the SNV 5’ UTR has been reiterated in other publications (Dangel et al., 2002; Roberts & Boris-Lawrie, 2000). Regions in the 5’ UTRs of human foamy virus and Mason–Pfizer monkey virus also facilitate cytoplasmic expression of their RNAs (Hull & Boris-Lawrie, 2002). A distinct mechanism of post-transcriptional control is exerted by the murine leukemia virus R region, which affects transport and expression of its target RNAs (Trubetskoy et al., 1999). Each of these divergent viruses has developed a way to overcome barriers to gene expression and, as discussed below, the method of nuclear export may affect cytoplasmic usage of viral RNAs (Boris-Lawrie et al., 2001; Dangel et al., 2002).

Candidate proteins associated with the Rev–RRE axis that may enhance translation

A large number of cellular proteins have been shown to influence Rev’s role in nuclear export (Askaer et al., 1998; Farjot et al., 1999; Fritz et al., 1995) and a number of candidates have been identified that could contribute to translational regulation (Table 1).

Eukaryotic initiation factor 5A (eIF5A) is a cellular interacting partner of Rev (Ruhl et al., 1993). The cellular role of eIF5A (previously known as eIF4D) is not yet clear (Zanelli & Valentini, 2007). It is the only known hypusine-containing protein and was originally thought to play a role in initiation of translation. UV cross-linking demonstrated an interaction between these two proteins and immunofluorescence showed that eIF5A, like Rev, has a nuclear localization. Subsequent experiments in Xenopus laevis and COS-1 cells showed that eIF5A is required for the Rev-dependent nuclear export of incompletely spliced RNAs. Ruhl et al. (1993) hypothesized that the interaction with eIF5A formed part of an RNP complex allowing cotranslocation of viral RNA and subsequent preferential translation, giving it both a nuclear and a cytoplasmic function. However, in these assays, an interaction was only found in the nucleus. Nevertheless, other eukaryotic initiation factors show nuclear localization (Lejkowsicz et al., 1992). Subsequently, it was shown that eIF5A localizes to the perinuclear region in an endoplasmic reticulum-like network and that this pattern was dispersed on dissociation of ribosomes by chemical treatment (Shi et al., 1997). The data on eIF5A were at first controversial; another group has failed to reproduce this interaction between eIF5A and Rev (Bogerd et al., 1995) and Xenopus oocytes were shown to be permissive for HIV-1 infection in the absence of exogenously added eIF5A (Fischer et al., 1994).

Subsequent publications, however, have demonstrated that eIF5A binds to the Rev–RRE complex specifically (Bevec et al., 1996). Mutant versions of eIF5A prevent HIV-1 replication in human CEM T cells and the activation domain of Rev is required for the interaction with eIF5A. Rev-specific indirect immunofluorescence microscopy demonstrated the requirement of eIF5A for nuclear export. Ribosomal protein L5 was found to interact with eIF5A through yeast two-hybrid assays. This was confirmed both by immunoprecipitation and coprecipitation from coupled in vitro transcription/translation reactions and from whole-cell lysates (Schatz et al., 1998). Coexpression of Rev and L5 in COS cells increased Rev-mediated p24 Gag protein levels in supernatants of cells transfected with proviral DNA, whilst neutralizing antibodies to L5 or eIF5A resulted in inhibition of Rev-directed export. L5 is involved in nuclear export of polymerase III transcripts and this contributes further to a model where eIF5A acts as an adaptor between the viral RNA and the cellular nuclear-transport machinery, allowing nuclear export. Interestingly, eIF5A is also a cofactor for HTLV-1 Rex (Katahira et al., 1995).

Poly(A)-binding protein 1 (PABP1, also called PABPC1, PABP, PAB1 or PBA) associates with cytoplasmic HIV-1 RNAs in a Rev-dependent manner (Campbell et al., 1994) and a number of studies carried out by the Jacobson laboratory (Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA, USA) suggest that the poly(A) tail has a role to play in translation. It has also been proposed that PABP1 may work with Rev to promote stability of viral RNAs (Bernstein et al., 1989; Felber et al., 1989). PABP1 has been implicated in the recruitment of ribosomes to eukaryotic mRNAs via the poly(A) region of the cytoplasmic tail (Kahvejian et al., 2005; Munroe & Jacobson, 1990; Tarun & Sachs, 1995). Purified PABP can stimulate translation in vitro (Sielianwanowicz, 1987),
Table 1. Cellular proteins that have been shown to interact with the Rev–RRE axis

Proteins known to be associated with Rev-dependent nucleocytoplasmic transport, e.g. CRM-1, are not included. Proteins discussed in the text are highlighted in bold. References for Sam68 are not listed, as the literature is extensive.

<table>
<thead>
<tr>
<th>Associated protein</th>
<th>Interaction</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>16.4.1</td>
<td>Interacts with Rev and CRM-1</td>
<td>Kramer-Hammerle et al. (2005)</td>
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<tr>
<td>B23</td>
<td>Interacts with Rev</td>
<td>Fankhauser et al. (1991)</td>
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<td>CK2</td>
<td>Interacts with and phosphorylates Rev</td>
<td>Meggio et al. (1996)</td>
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<td>DDX1</td>
<td>Interacts with Rev and the RRE; regulates Rev function and localization</td>
<td>Fang et al. (2005)</td>
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<tr>
<td>DDX24</td>
<td>Interacts with Rev; influences packaging of Rev-dependent RNAs</td>
<td>Ma et al. (2008)</td>
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<tr>
<td>DDX3</td>
<td>Transactivates Rev function and increases viral replication</td>
<td>Yedavalli et al. (2004)</td>
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<tr>
<td>elf5A (previously elf4D)</td>
<td>Interacts with Rev; required for Rev-dependent export</td>
<td>Bevec &amp; Hauber (1997); Bevec et al. (1996); Ruhl et al. (1993)</td>
</tr>
<tr>
<td>hnRNPA1</td>
<td>Interacts with L5 to enhance viral replication</td>
<td>Schatz et al. (1998)</td>
</tr>
<tr>
<td>hRIP/Rab</td>
<td>Interacts with Rev and enhances function and virus replication</td>
<td>Bogerd et al. (1995); Fritz et al. (1995); Sanchez-Velar et al. (2004); Yu et al. (2005)</td>
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<tr>
<td>IxB</td>
<td>Negatively regulates Rev function</td>
<td>Wu et al. (1993)</td>
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<tr>
<td>NFI90</td>
<td>Inhibits Rev function</td>
<td>Urcuqui-Inchima et al. (2006)</td>
</tr>
<tr>
<td>PABP1 (also PABPC1, PABP, PAB1, PBA)</td>
<td>Associates with HIV-1 RNAs in a Rev-dependent manner</td>
<td>Campbell et al. (1994)</td>
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<tr>
<td>Protein arginine methyl transferase 6 (PRMT6)</td>
<td>Methylates Rev</td>
<td>Invernizzi et al. (2006); Xie et al. (2007)</td>
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<tr>
<td>Prothymosin α</td>
<td>Interacts with Rev</td>
<td>Kubota et al. (1995)</td>
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<tr>
<td>Pur a</td>
<td>Interacts with Rev and RRE</td>
<td>Kaminski et al. (2008)</td>
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<tr>
<td>RNA helicase A (RHA)</td>
<td>Binds RRE in a Rev-dependent manner and increases expression</td>
<td>Li et al. (1999)</td>
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<tr>
<td>RREBP49</td>
<td>Binds RRE</td>
<td>Xu et al. (1996)</td>
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<tr>
<td>Sam68</td>
<td>Interacts with RRE and transactivates Rev function</td>
<td>See text</td>
</tr>
<tr>
<td>SF2/ASF</td>
<td>Binds RRE in a Rev-dependent manner</td>
<td>Powell et al. (1997)</td>
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<td>SLM-1, -2</td>
<td>Enhance Rev-mediated export</td>
<td>Reddy et al. (2002)</td>
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<tr>
<td>Tubulin</td>
<td>Rev depolymerizes microtubules</td>
<td>Watts et al. (2000)</td>
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<tr>
<td>YL2 (murine homologue of p32)</td>
<td>Interacts with Rev; transactivates RRE reporter expression</td>
<td>Luo et al. (1994)</td>
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<tr>
<td>β-Actin</td>
<td>Rev causes RRE-containing RNAs to colocalize with actin bundles</td>
<td>Tange et al. (1996); Kimura et al. (1996, 2000)</td>
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probably through PABP-interacting protein 1 (Paip1). Paip1 can interact directly with elf3 and form ternary complexes with PABP1 and elf4G (Martineau et al., 2008). Participation of the poly(A) tail in translation through PABP1 is now well established and may involve interactions with elf4G (Kessler & Sachs, 1998), elf4B (Bushell et al., 2001) and elf3 (Cosson et al., 2002), as well as other translation factors. PABP1 can also bind to an inhibitory RNA element within the coding region of the p17polyg matrix protein of HIV-1 (Afonina et al., 1997), possibly contributing to regulation of HIV-1 gene expression.

RNA helicase A (RHA) binds to the RRE in a Rev-dependent manner and increases expression from RRE-containing reporters in the presence of Rev (Li et al., 1999). Although that study showed that the stimulation was at the level of RNA export, there is evidence that RHA can stimulate translation from highly structured 5’ UTRs (Hartman et al., 2006) and that RHA increases translation of both reticuloendotheliosis virus A through its 5’ post-transcriptional control element and gag RNA from human T-cell leukemia virus 1 (Bolinger et al., 2007). Polysomal association of viral RNAs was reduced in the absence of endogenous RHA. The authors proposed that interaction of RHA with conserved structural elements within these RNAs allows for an increase in translation of viral RNAs. In a recent review by Bolinger & Boris-Lawrie (2009), the authors cite unpublished data that downregulation of RHA in the context of an HIV-1 proviral infection reduces translation of gag. Data from this laboratory suggest that...
the R and U5 regions of the 5′ UTR are involved in this [C. Bolinger, A. Sharma, P. Singh & K. Boris-Lawrie, unpublished data in Bolinger & Boris-Lawrie (2009)]. RHA shuttles between the nucleus and the cytoplasm and shows a steady-state nuclear localization (Tang et al., 1999). RHA may increase the efficiency of HIV-1 replication in a number of ways, including translation, and was identified as a cofactor for HIV in a recent screen (Brass et al., 2008).

The cellular protein Purz was recently shown to enhance the ability of Rev to stimulate expression from a luciferase reporter containing the RRE (Kaminski et al., 2008). Residues 18–50 of Rev and 73–123 of Purz were shown to interact by glutathione S-transferase pull-down and Rev was associated with endogenous and exogenous Purz in the cytoplasm of transfected cells. Purz–RRE and Purz–RRE–Rev complexes could be immunoprecipitated from the cytoplasm of transfected cells. Direct binding of Purz to the RRE is important for the enhancement of Rev activity. Purz has been shown to associate with ribosomes in dendritic cells (Johnson et al., 2006; Li et al., 2001). Perhaps of note is that Purz has strong affinity for the sequence GCC(A)GGA(C), which is remarkably similar to the 5′ side of the Rev-binding loop in the HIV 5′ UTR (CGCAGGAC).

One cellular protein that has received a large amount of attention with respect to its link with Rev is Sam68, although the publications often do not agree. Sam68 (68 kDa Src-associated substrate during mitosis) (Fumagalli et al., 1994; Taylor & Shalloway, 1994) is a nuclear RNA-binding protein containing an hnRNP K homology (KH) domain, which is phosphorylated by Cdc2 (Chen et al., 1997; Resnick et al., 1997). In virus-rescue assays, this protein has been shown to increase expression from a CAT reporter through interaction with the RRE (Reddy et al., 1999). This transactivation was shown to be reliant on the ability of Sam68 to localize to the nucleus (Reddy, 2000). A mutant incapable of nuclear localization was shown to inhibit Rev–RRE-mediated transactivation and viral replication. Transactivation can occur in the absence of Rev, but a synergistic effect is observed when both proteins are present. Leptomycin B, which inhibits Rev-dependent export of RRE-containing RNAs (Wolff et al., 1997), had no effect on transactivation by Sam68, indicating that these proteins operate in distinct ways. Inhibition of Cdc2 reduced the activity of Sam68 by 70% and of Rev by 25%. Transdominant mutant Sam68 proteins were capable of reducing viral replication. Transactivation also occurs through the HIV-2 RRE, Rex and equine infectious anemia virus (EIAV) Rev on their targets (Reddy et al., 2000) and is inhibited similarly by the mutant described above.

Others have shown that Sam68-like proteins SLM-1 and -2 are also capable of transactivating expression of RRE-containing RNAs in the same way as Sam68 (Soros et al., 2001). None of these three proteins affected Rev subcellular localization, and they are unable to shuttle between the nucleus and the cytoplasm and do not affect stability or accumulation of unspliced viral RNAs. They are unable to induce cytoplasmic expression of unspliced RNAs in the absence of Rev. These results imply that the transactivating effect on RRE-containing RNAs is not achieved through nucleocytoplasmic shuttling of target RNAs. Confusingly, the results contradict those of another study indicating that Sam68 and SLM-1 can increase levels of RRE–CAT mRNA and this correlates directly with the transactivation observed (Reddy et al., 2002). Sam68, SLM-1, SLM-2 and homologous quaking (QK) I-5, 6 and 7 proteins all enhanced Rev-mediated export of mRNA both in CAT and in virus-rescue assays.

Downregulation of Sam68 in infected astrocytes was shown to be the cause of the Rev-dependent defect in replication in these cells (Li et al., 2002b). Sam68 is not downregulated in permissive cells, such as Jurkat T cells, CEM cells and peripheral blood mononuclear cells (PBMCs). In RRE–CAT reporter assays in astrocytes, Sam68 had little to no effect on expression, but in the presence of Rev, transactivation was increased by 49%, compared with only 6.8% in 293T cells. Expression of exogenous Sam68 in infected cells increased virus production and appeared to relocalize Rev from the nucleus to the cytoplasm; Rev–Sam68 colocalization was observed close to the nuclear membrane. This group also showed that antisense knockdown of Sam68 reduced virus production in 293T cells and virtually abolished it in infected T cells or PBMCs (Li et al., 2002a), due to a decrease in Rev-mediated viral gene expression. They showed that Sam68 is not a nuclear-shuttle protein and that it does not interact with the nuclear transport protein CRM-1. Fluorescence microscopy indicated that cells treated with antisense Sam68 showed reduced Rev nuclear export; the authors hypothesized that Sam68 was involved in Rev-mediated nuclear export of HIV-1 incompletely spliced RNAs. Contrastingly, Coyle et al. (2003) showed that Sam68 did not increase cytoplasmic RNA levels as much as p24 levels were increased and concluded that Sam68 acted at a translational level. They suggested that CAT reporter assays, where leaking of RNA from the nucleus to the cytoplasm might be occurring, could explain these differences.

Sam68 can contribute to increased 3′-end processing of HIV-1 RNAs (McLaren et al., 2004), but this does not lead to increased cytoplasmic accumulation of incompletely spliced RNAs, suggesting again that Sam68 may be acting on translation. However, this conflicts with a report (Modem et al., 2005) where knockdown of Sam68 by RNA interference (RNAi) led to a reduction of Rev-mediated export of both CAT and Gag reporters, manifested as a reduction in cytoplasmic unspliced RNA in both cases. The domains of Sam68 important for HIV-1 replication have been characterized through analysis of dominant-negative mutants (Zhang et al., 2005). This study revealed a central proline-rich domain as being able to inhibit replication of HIV-1 in the absence of the Sam68
nuclear-localization signal. This dominant-negative effect affected Rev transport specifically and was involved in self-oligomerization of Sam68. Dominant-negative mutants were able to partially relocalize endogenous Sam68 to the cytoplasm of infected cells. The authors suggest that Sam68 influences cytoplasmic processes of Rev, such as translation. Sam68 phosphorylation may play a role in its regulation in HIV-1 infection (Najib et al., 2005). HS1-associated protein XI (Hax-1) is an anti-apoptotic mitochondrial protein and has been shown to inhibit Rev-dependent expression from RRE-containing RNAs (Modem & Reddy, 2008). This inhibition is alleviated by Sam68. Recently, intriguing data indicate that Sam68 can influence translation of tat/rev and nef RNAs differentially. Sam68 mutants were shown to be able to suppress translation of nef RNAs, but not tat/rev RNAs (Henao-Mejia et al., 2009). The distinction of these two RNA populations appears to occur through recognition of the 3′ UTRs of nef RNAs.

This story is still incomplete, with evidence pointing to roles for Sam68 in nucleocytoplasmic transport and RNA processing, as well as translation. Sam68 was identified as a cellular factor of HIV in a recent screen (Konig et al., 2008). Other proteins from this family (STAR proteins) have also been shown to be translational regulators (Jan et al., 1999; Jones et al., 1996; Saccomanno et al., 1999).

The DEAD-box helicase DDX3 seems to be involved in Rev/CRM-1 activity (Yedavalli et al., 2004). When Rev was coexpressed with DDX3 in HeLa cells, expression from Rev-responsive reporter plasmids was increased by more than 5-fold by correction of a defect in the cytoplasmic: nuclear ratio of incompletely spliced viral transcripts, which was mirrored in Gag expression levels. DDX3 shuttles between the nucleus and the cytoplasm and binds both CRM-1 and Rev. DDX3 coimmunoprecipitated with nucleoporins and localized to the outer rim of nuclear pores in HeLa cells. Whilst wild-type DDX3 increased viral replication in both transfection and infection systems, a dominant-negative mutant reduced it, assayed by p24 production. DDX3 may act to unwind CRM-1 RNA targets, allowing their translocation through the nuclear pore. DDX3 may also affect translation, as discussed in a recent publication demonstrating its interaction with elf3 (Lee et al., 2008a).

The authors demonstrate cytoplasmic localization of DDX3 and show by RNAi that DDX3 is required for protein expression. Dpb5p (the yeast homologue of DDX3, which has been investigated more extensively) has not been linked to translation, but has been found to associate with cytoplasmic fibrils (Strahm et al., 1999).

**Rev and genome packaging**

In addition to the roles already mentioned, Rev is suggested to facilitate encapsidation of HIV-1 genomic RNA. Despite the large excess of cellular RNAs compared with viral RNAs in the cytoplasm of infected cells, the viral genomic RNA is selected specifically from this large pool. The RNA genome is selected for incorporation into budding virions through interactions of residues in the nucleocapsid domain of Gag with the packaging signal in the 5′ UTR (Berkowitz et al., 1996; D’Souza & Summers, 2005; Lever, 2007). Since the original identification of the HIV-1 packaging-signal site (Lever et al., 1989), the region termed stem–loop 3 has been the favoured site for initiation of packaging (Clever & Parslow, 1997; Harrison et al., 1998). However, this region is not sufficient for encapsidation, and other sequences/structures must play a role (Berkowitz et al., 1995; Lever, 2007).

The RRE was first implicated in directing encapsidation of viral RNAs when Richardson et al. (1993) identified a 1.1 kb fragment of the env gene (spanning the RRE) that appeared to be an important supplementary packaging signal, conflicting with previously published work using different vector systems (Hayashi et al., 1992; Poznansky et al., 1991). Subsequent work suggested a contribution to packaging, but failed to show that the RRE was essential (Kaye et al., 1995). More recent work has found that singly spliced RNAs with and without the RRE are packaged into virions with equal efficiency, albeit much less efficiently than genomic RNA (Houzet et al., 2007). Analysis of sequences required for optimal lentiviral vector encapsidation, however, showed clearly that there is no correlation between the amount of RNA in the cytoplasm, as a result of constitutive transport elements, and the amount that is packaged, and confirmed the importance of the RRE (Anson & Fuller, 2003). Following this, the Überla group (Brandt et al., 2007) published data suggesting that the Rev–RRE interaction contributes to the selection of full-length RNAs for encapsidation. Omission of either Rev or the RRE from proviral vectors had only a mild effect on cytoplasmic vector RNA levels, but reduced vector titres dramatically (Lucke et al., 2005). Brandt et al. (2007) found, in an HIV-1 vector transfection system, that the packaging efficiency and therefore vector titre were reduced to 3% in the absence of Rev, whereas the cytoplasmic RNA levels were reduced to only 44%. There was no difference in Gag processing or particle production in the presence or absence of Rev using this codon-optimized system, which lacks the 5′ UTR and the RRE. However, with a wild-type HIV-1 vector containing these features, no virus particles were produced in the absence of Rev, a reduction that could not be explained completely by the reduction in cytoplasmic RNA. Analysis of proviral constructs containing a point mutation in Rev showed a 4- to 12-fold enhancement of cytoplasmic genomic RNA, but a 500-fold increase in encapsidation. Rev-independent expression from a gag–pol expressor was normal. Tethering a nuclear-localization signal-deficient Rev to genomic RNA failed to enhance encapsidation; however, tethering genomic RNA to the TAP export factor could enhance packaging (K. Überla, personal communication). Here, as elsewhere, there is a suggestion that nuclear events may influence the efficiency with which genomes are packaged (Swanson et al., 2004).
Mutation of the Rev-binding loop within the 5' UTR of HIV-1 RNA (Greatorex et al., 2002) in a proviral clone reduced virus infectivity in Jurkat cells (Greatorex et al., 2006), characterized by a number of deficiencies including reduced RNA trafficking, reduced dimer stability and a 30-35% packaging defect. Rev may, through loop A, traffic RNA to a particular site in the infected cell to allow efficient packaging into virions, particularly because the translation and packaging pools of full-length RNA are identical in HIV-1 and -2 (Dorman & Lever, 2000). In a similar way, the avian sarcoma virus structured directed repeat (dr) influences both cytoplasmic accumulation and packaging (Aschoff et al., 1999; Sorge et al., 1983).

Rev protein can be found in visna/maedi virus particles, indicating that it may play a role in particle production (Mazarin et al., 1990). Additionally, the capsid protein of cowpea chlorotic mottle virus has an arginine-rich motif similar to that of HIV-1 Rev, which allows recognition and packaging of specific RNAs (Annmalai et al., 2005). However, HIV-1 Rev has never been found in virus particles.

The proximity of the 5' UTR Rev-binding site to the dimerization-initiation site might suggest a role for Rev in dimerization. A link between dimerization and packaging in HIV-1 has been suggested (D'Souza & Summers, 2005; Greatorex, 2004; Paillart et al., 2004; Russell et al., 2004), manifesting itself as a contribution to cytosolic trafficking and localization (Swanson & Malim, 2006). This potential role of Rev remains to be investigated; however, disruption of the Rev-binding loop in the 5' UTR did lead to an impaired dimer stability (Greatorex et al., 2006).

**Export to encapsidation: the black box**

Transport of RNAs by Rev (and other ligands) could somehow mark them for particular destinations within the cytoplasm, as suggested by previous studies (Braddock et al., 1994; Matsumoto et al., 1998; Swanson et al., 2004). Although the passage of the virus into and out of the cell is reasonably well understood, the journey that the unspliced RNA takes, having exited the nucleus, to its encapsidation at the cell membrane is by comparison obscure.

Gag produced from Rev-independent vectors localizes differently in the cytoplasm from Gag produced from Rev-dependent vectors, and Gag expressed from certain Rev-independent vectors fails to release virus particles from HeLa cells in an efficient manner (Swanson & Malim, 2006). Also, gag-pol RNAs trafficked through a constitutive transport element are not translated efficiently (Coyle et al., 2003) and are not associated with polysomes unless co-expressed with Tap/NXT (Jin et al., 2003). This further implies that the mode of export of RNAs from the nucleus determines their cytoplasmic fate.

In recent years, a number of studies have focused on the functional interactions between HIV-1 RNA, HIV proteins and cellular RNA helicases. DDX1 was shown to interact with Rev (Fang et al., 2004). Knockdown of DDX24 by small interfering RNA restricts virus infection (Ma et al., 2008), accompanied by a reduction in RNA packaging, but not of dimerization or viral RNA expression in infected cells. DDX24 colocalized with Rev in the nucleolus and co-purified with Rev from cell lysates. This interaction was partially RNase-resistant, further supporting a direct interaction between these two proteins. Surprisingly, DDX24 knockdown does not impact negatively on Rev-dependent RNA export; in fact, a slight increase was observed, accompanied by a similar increase in viral protein expression. However, DDX24 knockdown reduces packaging of Rev-mediated RNAs exported from the nucleus, consistent with the data of Brandt et al. (2007).

 Trafficking of RNAs by Rev is important for allowing Rev to target its bound RNAs to the polysomes (Arrigo & Chen, 1991; D’Agostino et al., 1992; Jin et al., 2003). If Rev is able to direct both translation and packaging of its target RNAs, how is the fate of these RNAs decided? Temporal and spatial factors are likely to be important in answering this question, as is the influence of other viral proteins, particularly Gag. Importantly, Gag is the only viral protein required for virus-particle assembly (Krausslich & Welker, 1996). Gag colocalizes with full-length viral RNAs in the perinuclear region in the vicinity of the centrioles, a localization that is dependent on the packaging signal being intact (Kimura et al., 1996; Poole et al., 2005), and it has been proposed that Gag–full-length RNA complexes may associate with P bodies, where these complexes would be removed from competition with ribosomes and so directed towards packaging (Swanson & Malim, 2006).

**Other lentiviral Rev proteins**

All lentiviruses and deltaretroviruses express proteins functionally analogous to HIV-1 Rev, although the conservation of the nuclear-export role does not necessarily mean that they are identical in all of their other actions (Cullen, 1992).

Like HIV-1, HIV-2 and simian immunodeficiency virus (SIVmac) both encode Rev proteins that are able to induce cytoplasmic expression of incompletely spliced RNAs (Malim et al., 1989a). HIV-2 Rev (Rev2) localizes to the nucleus of transfected cells and has a basic region in the amino-terminus that is required for nuclear localization and function. Unlike HIV-1 Rev, it is not phosphorylated efficiently (Dillon et al., 1991), although the relevance of this remains to be determined. HIV-1 Rev is able to transactivate through the HIV-2 RRE; however, this is not reciprocated (Malim et al., 1989a; Sakai et al., 1991). Confusingly, Rev2 was shown to be both able (Garrett & Cullen, 1992) and unable (Dillon et al., 1990; Sakai et al., 1991) to bind to the HIV-1 RRE. SIVagm can also be transactivated through the Rev proteins of HIV-1, HIV-2 and other SIV strains (Sakai et al., 1991). The functional specificity appears to be mediated through the second exon of Rev (Furuta et al., 1995).
Visna/maedi virus encodes a functionally equivalent protein, Rev-V (Sargent & Bennet, 1989), that is required for productive viral infection (Toohey & Haase, 1994). This localizes to the nucleoli of infected and transfected cells (Schoborg & Clements, 1994), but is also found in the cytoplasmic fraction of infected cells (Mazarin et al., 1990). Rev-V, like HIV-2 Rev but unlike HIV-1 Rev, is not phosphorylated efficiently (Schoborg & Clements, 1994). Rev-V transactivates through a responsive element in the env open reading frame, but this shows no similarity to the HIV-1 or 2 RREs and Rev-V is unable to transactivate through these sequences. Similarly, HIV-1 Rev is unable to transactivate through the visna/maedi virus RRE (Tiley & Cullen, 1992). Rev-V has the same functional domain organization as Rev of HIV-1, to the extent that chimeras between the two proteins are fully functional in domain-swap experiments (Tiley et al., 1991).

EIAV encodes a Rev protein (Stephens et al., 1990) that, although functionally equivalent to those of other retroviruses, appears to have a different domain organization (Fridell et al., 1993; Mancuso et al., 1994). As well as nuclear export, EIAV Rev (ERev) appears to have a role in alternative RNA splicing of its own RNA and overcomes inhibitory effects of splice sites (Martarano et al., 1994; Rosin-Arbesfeld et al., 2000; Tan et al., 1996), as described for HIV-1 (Afonina et al., 1997; Cochrane et al., 1991; Maldarelli et al., 1991; Schneider et al., 1997; Schwartz et al., 1992; Tarun & Sachs, 1995). This Rev protein responds to two sequences in the viral RNA, and sequence variation/mutation within ERev alters the ability of ERev to mediate export through these sequences, but not alternative splicing, suggesting that these are separable activities (Baccam et al., 2003; Belshan et al., 1998; Harris et al., 1998; Sparks et al., 2008). However, subsequent studies show that mutation of the exon-splicing enhancer through which ERev modulates alternative splicing also reduces nuclear export (Belshan et al., 2000). Splicing factor SF2/ASF has been shown to bind to the EIAV RRE (ERRe) and it is thought that simultaneous binding of the two proteins regulates splicing from the tat/rev RNA (Chung & Derse, 2001). The interaction of ERev with its ERRe appears to occur through a bipartite RNA-binding motif in the second exon (Lee et al., 2006). Although the EREV exhibits structural differences from those of other lentiviruses, a recent paper investigating the secondary structure and Rev-binding properties of the ERRe revealed a conserved structural motif between the ERRe and the RREs of other, diverse lentiviruses (Lee et al., 2008b).

A functional equivalent of Rev is found in feline immunodeficiency virus (FIV), which transactivates through a highly structured region at the end of the env open reading frame (Kiyomasa et al., 1991; Phillips et al., 1992). FIV Rev is able to transactivate CAT reporter expression in feline cells, but not non-feline cells (Tomonaga et al., 1994). Transactivation occurs through a short effector domain that differs from those of other lentiviruses (with the exception of EIAV), but is still sensitive to leptomycin B inhibition (Mancuso et al., 1994; Otero et al., 1998). Members of the T-cell leukemia virus subgroup all encode functional Rev proteins, with HTLV-1 Rev having a domain organization distinct from that of HIV-1 Rev (reviewed by Cullen, 1992).

Given the fact that lentiviral Rev proteins exhibit a degree of variation, including domain organization, sequence specificity and in some cases functionality, it is not surprising that additional roles of Rev could exist in this cohort of proteins. The complexity and mechanism of control of gene expression in these viruses vary, but the essential nucleocytoplasmic-export function of their Rev protein homologues is conserved. Investigation into the function of other lentiviral Revs has been largely limited to nuclear transport, and so future work may reveal additional functions of these proteins. The fact that ERev is able to regulate alternative splicing from its own RNA illustrates that, whilst being a conserved protein, Rev has acquired different functions in divergent lentiviruses.

Conclusions

There is now a considerable body of evidence that Rev is able to influence translation and encapsidation of viral RNAs. One possible mechanism may be by directing subcellular trafficking of RNAs to an appropriate location within the cytoplasm, towards either a packaging pathway or the translation machinery, probably assisted by differential interactions with cellular proteins. The fact that Rev can stimulate translation in an in vitro system (Groom et al., 2009), however, implies in addition a more direct effect on stimulating translation.

How might the two Rev-binding sites in HIV-1 RNAs contribute to these phenomena? The RRE is contained only on incompletely spliced RNAs and may enhance selection of full-length RNAs for encapsidation. In contrast to this, the 5’ UTR Rev-binding site is found on all viral RNAs and might effect preferential translation of viral versus cellular transcripts. Further analysis of the roles played by these two binding sites in the functions of Rev will provide insight into the complexity of this system.

Rev has a major role in the early-to-late switch in the productive phase of the virus life cycle. All of these additional functions are coherent with this and allow it to exert even finer control over coordinated expression of viral genes, a function that probably contributes to immune evasion, as well as translation and packaging of viral components. A report addressing the effect of splicing and transport on the dynamics of computer-modelled HIV-1 infection underlined the complex and sensitive nature of control of gene expression in HIV-1 (Kim & Yin, 2005). Knowledge of the modes of action of Rev and the variations between lentiviruses provides a series of model systems for understanding cellular gene expression. Each additional role that is uncovered makes this protein more pivotal as a therapeutic target (Chaloin et al., 2007; Jin &
Cowan, 2006; Mills et al., 2006; Moeble et al., 2007; Shuck-Lee et al., 2008; Ye & Li, 2006). In the face of rapid development of resistance to current drug regimes (Hirsch et al., 2008; Johnson et al., 2008), research on Rev is far from over.

References


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Rev: beyond nuclear export


