RNA export: insights from viral models

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Introduction

A defining feature of eukaryotic cells is the division of the cell into a nucleus and cytoplasm. This division separates the site of RNA transcription from the site of translation, and this requires mRNAs to be exported from the nucleus via the nuclear pore to direct the synthesis of proteins in the cytoplasm.

Importantly, naked RNA is not exported. In the nucleus, RNA is associated with a number of proteins forming a ribonucleoprotein (RNP) complex. RNPs exit the nucleus by going through the nuclear pore complex, which is also composed of a large number of proteins and functions as a specific gate. Only designated proteins or RNPs are allowed to pass through the nuclear pore. Identification of the proteins involved in export – both those that bind or are associated with the RNA and those in the nuclear pore complex – is ongoing.

Experiments in Xenopus oocytes reveal multiple, energy-dependent export pathways for different classes of RNA [1]. In these experiments, radiolabelled RNAs are microinjected into the nuclei of Xenopus oocytes. After incubation, nuclei are dissected manually from the cytoplasm and RNA is extracted from both compartments. Rates of export can be determined by the relative amounts of RNA in each compartment over time. Injection of an excess of unlabelled RNA will inhibit export, presumably by saturating a limiting factor. For example, if an excess of mRNA is used, then radiolabelled mRNA will

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Figure 1. Pathways of RNA export
(a) Facilitated RNA export by the Rev protein of complex retroviruses. The integrated retrovirus genome, flanked by long terminal repeats (LTR), is transcribed to make an unspliced genomic RNA. This RNA contains two introns. The genomic RNA can be spliced to remove one or both introns. The completely spliced RNA is exported via the normal mRNA-export pathway. Intron-containing transcripts must be exported by a Rev-facilitated pathway. The intron-containing
(contd.)
not be exported but other radiolabelled RNAs continue to be exported. These competition experiments reveal multiple RNA-export pathways for mRNA, tRNA, small nuclear RNA (snRNA) and rRNA.

Additionally, it should be noted that a lack of export does not necessarily mean that export itself is defective. RNAs undergo co-transcriptional and post-transcriptional processing in the nucleus. Some of these events are prerequisites for RNA export. Thus a defect in a processing step might appear as an export defect.

Studies of the transport of unconventional viral RNA have shed light on the complicated process of RNA export. The first unconventional viral RNAs studied were those that were exported with introns. In normal RNA export, introns are removed by splicing before export can take place. In contrast, retroviruses export RNA containing one or more introns. One class, the complex retroviruses, do so using a protein called Rev (Figure 1a). Key experiments were possible with this system because Rev was virally encoded. Stimulated by the success of these studies, another class of retroviruses, the simple retroviruses, which also transport an intron-containing RNA into the cytoplasm but do not encode a viral protein to mediate this process, began to receive attention. RNA elements important for the cytoplasmic localization of the unconventional RNA were discovered. Recently, a protein that binds one of these elements and facilitates export has been identified.

Finally, another kind of viral RNA is being studied as a potential new model for RNA export. Herpes simplex virus (HSV) and hepatitis B virus (HBV) produce intronless RNAs. Cis-acting RNA elements have been identified in these transcripts that affect the cytoplasmic accumulation of several different transcripts. These elements are believed to act at the level of RNA export.

This chapter is divided into three major sections. In the first section, we discuss viral RNA export mediated by Rev or proteins with a related domain. The second section focuses on export of intron-containing RNA that uses cellular proteins that are not related to Rev. The third and last section is devoted to cis-acting RNA elements involved in the export of intronless viral transcripts.

Figure 1. Pathways of RNA export (contd.)

transcripts contain the Rev-responsive element (RRE) within the second intron. Multiple Rev molecules bind the RRE. A complex between Rev, RRE, CRM1 and Ran results in the export of the bound RNA. In the nucleus, Ran binds GTP. This form of Ran is required for export. (b) Facilitated export of intron-containing RNA of a simple retrovirus. Like the complex retroviruses, the Mason–Pfizer monkey virus genome is transcribed to make an unspliced genomic RNA. In contrast, this RNA only contains a single intron. The genomic RNA can splice to generate a product that is exported via the normal mRNA-export pathway. The constitutive transport element (CTE) is located near the 3′ end of the viral transcripts. The CTE facilitates the export of the intron-containing RNA. This facilitated pathway uses the cellular protein TAP, which binds the CTE, rather than a virally encoded protein. TAP is also involved in normal mRNA export, but its precise role has not been determined.
RNA export mediated by Rev or Rev-like proteins

The role of the Rev proteins in export was first suggested by genetic experiments with human immunodeficiency virus (HIV). HIV encodes its structural, enzymic, regulatory and accessory proteins from a compact genome of less than 10 kb. The virus uses post-translational cleavage, overlapping reading frames, alternative splicing and intronic messages to express all of these proteins. The structural and enzymic proteins are expressed from the full-length genomic transcript that contains two introns. The envelope protein is expressed from a partially spliced, single intron message. Translation of these transcripts and packaging of the viral RNA genome in the cytoplasm requires that these intron-containing RNAs bypass the normal requirement for splicing before export. In a Rev-minus virus, the intron-containing RNAs are not observed in the cytoplasm, while completely spliced messages, including the Rev transcript, accumulate in the cytoplasm. Rev binds to a specific RNA element, the Rev-responsive element (RRE), located in the second HIV intron. It was hypothesized that once bound to the RRE, Rev facilitated the export of the intron-containing RNA (Figure 1).

At steady state, Rev is observed in the nucleus. A nuclear localization signal, a short peptide motif in the protein, confers this localization. However, Rev was shown to move continuously between nucleus and cytoplasm in the following way. Human cells expressing Rev were fused to non-expressing mouse cells to form multi-nucleated cells or heterokaryons. Before fusion, Rev was localized in the nuclei of the human cells. After fusion, Rev was found in both the human and mouse nuclei. This assay demonstrated that Rev was a shuttling protein [2]. By moving between nucleus and cytoplasm, Rev could redistribute into both human and mouse nuclei in the heterokaryon cell (Figure 2). Such experiments illustrate the disadvantage of looking at protein localization as a static picture. Rev’s nuclear localization reflects its equilibrium distribution but belies the fact that it shuttles. Importantly, Rev shuttles in the absence of its RNA substrate [2], lending credence to the idea that Rev is involved in exporting RNA.

To test Rev’s role in RNA export, Rev and an RNA substrate were microinjected into oocyte nuclei. The substrate RNA consisted of an intron containing the RRE flanked by two exons. Splicing of the RNA occurred in the nucleus, but remarkably Rev was able to export the excised intron, known as a lariat because of its structure, following excision. The lariat, normally a highly unstable nuclear RNA, is more stable in oocyte nuclei. Export of the lariat by Rev demonstrated that Rev could act after splicing [3]. This dispelled the idea that Rev functioned by masking the presence of the intron within the RNA. Moreover, these experiments were direct evidence that Rev exported RNA.
Rev contains a nuclear export sequence (NES)

The N-terminus of Rev contains the nuclear localization signal, RNA-binding domain and a multimerization domain. The C-terminus of Rev contains a leucine-rich sequence that is required for function. The leucine-rich sequence was demonstrated to be a NES by microinjection studies [4,5]. A fusion protein consisting of glutathione S-transferase (GST) and the leucine-rich peptide was expressed in bacteria, purified and injected into the nuclei of somatic cells [5]. The GST-NES protein, but not GST, was rapidly exported to the cytoplasm. Similarly, the NES peptide conjugated to BSA, but not BSA alone, was exported when injected into the nuclei of *Xenopus* oocytes or somatic cells [4]. In both systems, conjugated peptides with mutations in the leucine-rich sequence remained in the nucleus. This demonstrated for the first time that the leucine-rich sequence conferred nuclear-export properties to Rev.

Along with the discovery of a NES in Rev, Wen et al. [5] showed that the cellular protein kinase A inhibitor (PKI) had an NES. A growing number of cellular proteins with Rev-like NESs are being identified; we list only a few examples in Table 1. Other large hydrophobic residues as well as leucines characterize these NESs (known or potential key hydrophobic residues in the NESs are shown in bold). Note that these hydrophobic residues adopt a similar but not identical spacing pattern in all the NESs. NES-bearing proteins are not limited to RNA export; they include cell-cycle regulators, transcription factors and structural proteins. It is thought that by mimicking a signal found in cellular proteins, Rev can interact with cellular machinery responsible for export. The discovery of the Rev NES made it possible to study and define that export machinery.
Table 1. Structure of nuclear export signals

<table>
<thead>
<tr>
<th>Abbreviations used: EBV, Epstein-Barr virus; HTLV, human T-cell leukaemia virus</th>
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<tbody>
<tr>
<td><strong>Viral NESs</strong></td>
</tr>
<tr>
<td>HIV-1 Rev</td>
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<tr>
<td>HTLV-I Rex</td>
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<td>HSV ICP27</td>
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<td>EBV Mta</td>
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<td>Influenza A NS2</td>
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<td>Influenza B NS2</td>
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<td><strong>Cellular NESs</strong></td>
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<tr>
<td>PKI</td>
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<tr>
<td>p53</td>
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<tr>
<td>Map kinase kinase</td>
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<tr>
<td>c-abl</td>
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<tr>
<td>cyclin B1</td>
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<tr>
<td>α-Actin (NES1)</td>
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<tr>
<td>α-Actin (NES2)</td>
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**Rev accesses a specific RNA-export pathway in the cell [4]**

RNA export is a saturable process [1]. Microinjection of oocyte nuclei with an excess of unlabelled NES-conjugated BSA inhibited the export of labelled conjugates. Export of different RNAs was then tested under these inhibiting conditions. Excess NES-conjugated BSA inhibited the export of U snRNAs and 5 S rRNAs but had no effect on mRNA or tRNA export. Thus, Rev-mediated export uses cellular machinery that exports U snRNAs and 5 S rRNA. The transcription-factor protein TFIIIA, known to be involved in 5 S rRNA export, was shown subsequently to contain a Rev-like NES.

A significant advance in the RNA-export field was the identification of the Rev nuclear-export-signal receptor [6,7]. It was hypothesized that for Rev to carry out its function, a cellular protein must recognize and interact with the NES. Leptomycin B (LMB), an anti-microbial drug, was the key to identifying the NES receptor. Treatment of cells with LMB specifically blocks the export of Rev-dependent HIV RNAs but not the Rev-independent transcripts [8]. LMB also inhibits the export of Rev and U snRNAs in oocytes [6]. Furthermore, LMB inhibits the growth of the baker’s yeast *Saccharomyces cerevisiae* but not the fission yeast *Schizosaccharomyces pombe*. LMB resistance in *S. pombe* maps to a gene called *CRM1* or chromosome maintenance region 1. In vitro, Rev binds CRM1, and this binding is dependent on a functional NES in Rev; LMB disrupts this interaction [6,7]. These data suggest that CRM1 is the NES receptor. If CRM1 is the limiting factor in oocyte nuclei, then excess CRM1 should stimulate Rev and U snRNA export and reverse the inhibition of LMB. These predictions were found to be true and defined
CRM1 as a key factor in NES-dependent export [6]. CRM1 is also known as exportin 1 to emphasize its role in export.

CRM1 is a member of the β-importin superfamily. Members of this super-family have in common a domain that binds the small GTPase Ran. Ran is a key factor in the bi-directional transport of macromolecules between the cytoplasm and nucleus. Import and export complexes, including the CRM1–Rev complex, interact with Ran (Figure 1). Binding of GTP or GDP to Ran sends signals to transport complexes revealing whether they are in the nucleus or cytoplasm.

Other viruses encode Rev-like proteins. For example, human T-cell leukaemia virus encodes a NES-containing protein called Rex that binds to an element in the viral long terminal repeats. ICP27 from HSV, Mta from Epstein–Barr virus and non-structural protein 2 (NS2) or NEP from influenza virus have all been reported to encode NES-containing proteins [9–11] (Table 1). ICP27 appears to export intronless RNA [10,11]. Interestingly, CRM1 does not appear to be involved in cellular mRNA export.

**Rev-independent facilitated export**

Simple retroviruses, like the complex retroviruses, must bypass the splicing requirement for export, but there is one important difference (Figure 1b). These viruses do not encode a viral protein involved in RNA export. Instead, their RNAs contain binding sites for cellular export proteins. Studies of these viruses have focused on the identification of a cis-acting RNA element required for intron-containing export in the type-D retroviruses. An element was found in the Mason–Pfizer monkey virus (MPMV) and the related simian retroviruses types 1 and 2. This element, known as the constitutive transport element (CTE), has a key role in the export of cellular RNA-export proteins. Similar elements in Rous sarcoma virus, an avian retrovirus, and murine leukaemia virus that mediate cytoplasmic accumulation of the genomic RNAs have been identified [12,13].

**The CTE is a small RNA sequence located at the 3’ end of MPMV [14]**

Using mutagenesis and nucleic acid-modifying reagents, a secondary structure in the CTE was identified [15]. The CTE consists of two stem loops, one on top of the other. The loops have the same primary sequence but are rotated 180° relative to each other and are required for function (Figure 3). The loops were proposed to be the binding site for a cellular factor that mediates function. While the CTE is present in both the unspliced and spliced viral RNAs (Figure 3), it is only required for export of the unspliced transcript [16].

The evidence that the CTE was a genuine RNA-export element again came from microinjection of *Xenopus* oocytes [17]. The CTE was placed in the intron of the same construct used to test Rev and the RRE. RNA transcribed from this construct was microinjected into oocyte nuclei. Like Rev, the CTE
caused export of the lariat. Therefore the CTE was then tested for its ability to inhibit other RNA export.

The CTE uses a factor involved in mRNA export
When excess CTE RNA was microinjected into nuclei, it competed with the export of radiolabelled mRNA [17]. Export of radiolabelled U snRNA, tRNA and Rev-dependent RNA was not inhibited, nor was CTE export inhibited by an excess of NES-conjugated BSA. These results are consistent with the CTE using the mRNA pathway. Furthermore, CTE export is not sensitive to LMB [18]. Therefore the CTE and Rev both export intron-containing viral RNA, but they do so via two different export pathways.

The protein TAP binds the CTE and mediates CTE-dependent RNA export
Using a radiolabelled CTE RNA, a protein from nuclear extract that bound to the CTE was identified [19]. This protein is TAP, the human homologue of the yeast protein Mex67p. Mex67p is implicated in mRNA export in yeast. TAP
binds specifically to the CTE in that it does not interact with CTE loop mutants that are deficient for export of the lariat structure from oocyte nuclei. As discussed above, excess CTE RNA can titrate a limiting factor for export of mRNA from oocytes. However, mRNA export could be restored by microinjection of TAP. This demonstrates that TAP is probably the saturable factor for CTE and mRNA export in oocytes (Figure 3). RNA helicase A is also reported to bind the CTE [20]. Its role, if any, in export of MPMV RNA is not known.

Export of intronless viral RNA

Several viruses including HBV and HSV produce intronless transcripts. Not only do introns serve as retention signals until splicing is complete, but introns also have positive effects on RNA processing. When introns are inserted into cDNAs (a gene with the introns removed) and the transcript is transfected into cells, the resulting protein expression is often increased. Analysis of some of these introns reveals that they stimulate 3′-end formation of messages. 3′-End formation, which consists of cleavage of the RNA transcript followed by the addition of a polyadenosine tail, indirectly affects export because it is a prerequisite for transport. For example, the β-globin transcript requires an intron for 3′-end formation and subsequent transport to the cytoplasm. The process of splicing may also play a role in assembling factors on to the RNA for export or for other RNA-processing events. Thus intronless messages may compensate for the lack of an intron with specialized RNA elements.

HSV-1 is a nuclear-replicating DNA virus. Of more than 70 HSV-1 genes, only four of those expressed during lytic infection contain introns. Early studies of one intronless transcript, the thymidine kinase (TK) message, revealed two interesting observations. First, the TK transcript could overcome the β-globin transcript’s requirement for an intron. Secondly, intron-containing chimaeric transcripts could be found in the cytoplasm with TK.

An RNA element was found in the intronless TK transcript. Liu and Mertz [21] mapped a 119-nucleotide element within the TK transcript that could rescue expression of a β-globin transcript lacking introns. This element, the pre-mRNA processing enhancer (PPE) was found to bind heterogenous nuclear (hn) RNP L [21]. Mutations of the PPE were used to establish a correlation between binding and cytoplasmic accumulation of β-globin.

One proposed mechanism for the PPE of TK was stimulation of 3′-end processing [21]. The ability of the PPE to replace an intron in β-globin argues that it has the same function as the intron to ensure efficient 3′-end formation of β-globin (see above). The observation that TK could cause intron-containing RNA to accumulate in the cytoplasm suggested an export mechanism. Otero and Hope [22] found that the TK transcript can replace Rev and the RRE in the export of unspliced HIV RNA to the cytoplasm. Could the TK transcript be mimicking the RRE by binding a Rev-like cellular factor? Otero
and Hope demonstrated further that, in this assay, TK was insensitive to LMB [22]. This indicated that the TK transcript did utilize the Rev-export pathway. Nevertheless, the TK transcript could contain an RNA-export element. The relationship between the CTE and the TK-transcript export pathways remains to be determined. Recently, Huang et al. [23] found the PPE increases 3’-end formation and allows for the transport of a β-globin construct lacking an intron. This finding supports both proposed mechanisms for the PPE. Liu and Mertz [21] suggested and Otero and Hope [22] demonstrated that there are additional cis-acting elements in the TK transcript besides the PPE that are involved in RNA export to the cytoplasm. This finding raises the possibility that multiple functions are carried out by distinct elements within the intronless TK transcript.

A functionally similar element, known as the post-transcriptional regulatory element (PRE), has also been identified in the HBV [24]. This element, like the HSV TK coding sequence, consists of multiple cis-acting sequences, can facilitate the export of an intron containing RNA and stimulate 3’-end formation, and functions in an LMB-insensitive manner [18,23]. Interestingly, the PRE in the closely related woodchuck hepatitis virus (WPRE) has the ability to post-transcriptionally stimulate the expression of heterologous cDNAs up to 10-fold [25]. This ability is unique among all the viral elements discussed in this chapter.

Relevance and future goals

Studies of these elements have focused on how these viral RNAs bypass normal cellular requirements. In studying this, we illuminate normal cellular processes and generate ideas for practical applications of gene expression. Experiments with Rev and the CTE have helped define RNA-export pathways and cellular proteins involved in export. In addition the intronless RNA elements are capable of increasing gene expression, which should be of general interest to researchers.

The post-transcriptionally acting viral RNA elements that we have discussed are summarized in Table 2, together with others not mentioned here. Questions about mRNA export remain. What role does TAP play in mRNA export? Are there subsets of mRNA that use different factors for export? For intronless transcripts, the mechanisms by which the RNA elements act remain to be determined. Studying these transcripts begs the question of the positive functions of introns. For example, do introns assemble export factors that can act after splicing? Identifying the proteins that bind RNA elements from intronless transcripts may shed light on this question. Further, these elements may teach us more about RNA processing.
Table 2. Export of unconventional viral RNAs

(1) The post-transcriptional elements in TK function without ICP27 and are not sensitive to LMB. However, ICP27 should be sensitive to LMB because it contains a Rev-like NES.
(2) NS2 has not been shown to bind RNA directly. The viral protein M1 probably bridges NS2 and RNA, but NS2 may interact with the RNA via other proteins.
(3) These proteins should be sensitive to LMB because they contain a Rev-like NES, but the experiments have not been done.
(4) Five Epstein-Barr virus replication genes have been shown to require Mta for RNA export; other viral genes may also be dependent on Mta. Mapping of a specific element, if it exists, is in progress.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Unconventional RNA</th>
<th>RNA element</th>
<th>RNA binding</th>
<th>Is export sensitive to LMB?</th>
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<tbody>
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<td>Complex retroviruses: HIV</td>
<td>Intron-containing</td>
<td>Rev responsive element</td>
<td>Rev (viral; has a NES)</td>
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<tr>
<td>Type D retroviruses: MPMV</td>
<td>Intron-containing</td>
<td>Constitutive transport element</td>
<td>TAP (Cellular)</td>
<td>No</td>
</tr>
<tr>
<td>Mammalian HBV</td>
<td>Intronless</td>
<td>Post-transcriptional regulatory element</td>
<td>Unknown</td>
<td>No</td>
</tr>
<tr>
<td>HSV</td>
<td>Intronless</td>
<td>Pre-mRNA processing enhancer and additional elements in TK</td>
<td>ICP27 (viral; has a NES) and hnRNP L (cellular)</td>
<td>No (1)</td>
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<tr>
<td>Influenza virus</td>
<td>Non-polyadenylated, non-messenger, genomic RNA</td>
<td>See (2)</td>
<td>NS2 [viral; has a NES; see (2)]</td>
<td>Yes (3)</td>
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<tr>
<td>Epstein-Barr virus</td>
<td>Intronless</td>
<td>See (4)</td>
<td>Mta (viral; has an NES)</td>
<td>Yes (3)</td>
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</tbody>
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Summary

- The retroviruses export intron-containing RNA.
- The complex retroviruses encode a Rev protein that uses a leucine-rich NES to interact with CRM1 and the U snRNA-export pathway.
- Other viruses encode proteins with a Rev-like NES.
- The type-D retroviruses contain a CTE that binds the cellular protein TAP to export intron-containing RNA through the mRNA pathway.
- Intronless viral transcripts contain post-transcriptionally acting RNA elements that may compensate for the lack of an intron.
- The functions of elements in intronless RNA are not fully understood but may be in export and/or 3'-end processing.

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References