RNA-binding Motif Protein 15 Binds to the RNA Transport Element RTE and Provides a Direct Link to the NXF1 Export Pathway*

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Retroviruses/retroelements provide tools enabling the identification and dissection of basic steps for post-transcriptional regulation of cellular mRNAs. The RNA transport element (RTE) identified in mouse retrotransposons is functionally equivalent to constitutive transport element of Type D retroviruses, yet does not bind directly to the mRNA export receptor NXF1. Here, we report that the RNA-binding motif protein 15 (RBM15) recognizes RTE directly and specifically in vitro and stimulates export and expression of RTE-containing reporter mRNAs in vivo. Tethering of RBM15 to a reporter mRNA showed that RBM15 acts by promoting mRNA export from the nucleus. We also found that RBM15 binds to NXF1 and the two proteins cooperate in stimulating RTE-mediated mRNA export and expression. Thus, RBM15 is a novel mRNA export factor and is part of the NXF1 pathway. We propose that RTE evolved as a high affinity RBM15 ligand to provide a splicing-independent link to NXF1, thereby ensuring efficient nuclear export and expression of retrotransposon transcripts.

General mRNA export in eukaryotes is mediated by NXF1. Most mRNAs leave the nucleus in their unspliced form whereas pre-mRNA is generally retained in the nucleus due to the lack of active export and to factors retaining the pre-mRNA in the nucleus. Retroviral transcripts are a notable exception from this rule, because the unspliced transcript encoding the gag-pol polyprotein and also serves as viral genomic RNAs, and, therefore, needs to be exported prior to splicing. To overcome the general requirement of splicing before export, simian Type D retroviruses and some retroelements utilize the constitutive transport element (CTE) (29–33) that serves as a high affinity NXF1 ligand (3), thereby providing constitutive, splicing-independent export signals.

We had discovered and characterized a novel family of cis-acting RNA transport elements (RTEs) that are present abundantly in the mouse genome and are associated with intracisternal A-particle retroelements (IAP) (34, 35). RTE is functionally analogous to CTE, yet structurally unrelated, and does not bind to NXF1 with high affinity. In this work, we report the identification of the factor that directly promotes RTE function.
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We report that the RNA-binding motif protein 15 (RBM15) recognizes RTE RNA specifically in vitro and activates export and expression of RTE-containing reporter mRNAs in vivo. RBM15 also binds to NXF1, and these factors act cooperatively in promoting RTE-mediated expression. Thus, RBM15 is a novel component of the NXF pathway.

MATERIALS AND METHODS

Recombinant DNA—The reporter plasmids pNLgag, pNL-Cag, or pDM138 containing RTE, mutant RTE, CTE, or RRE (34–38), pDM128/PL and pDM128/B (39), the CMVgag/pol plasmids containing a polylinker or the MPMV-CTE (40), the CMVgag, or pDM138 containing RTE, mutant RTE, CTE, or RRE was obtained from S. Morris, and the FLAG tag was removed. The immobilized RNAs were incubated with micrococcal endonuclease-treated nuclear HeLa cell extract in buffer RBB (15 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, and 0.2% Triton X-100) with 300 mM NaCl (RBB-300) supplemented with 4 μg/ml tRNA and 2 μg/μl tRNA in 400-μl reactions at 30 °C for 2 h. The beads were washed 6× in RBB-300, and the bound proteins were eluted from the RNAs in 1 M NaCl, separated on a 10% SDS-PAGE, and visualized by silver staining.

In-gel Tryptic Digestion, Nano-HPLC Separation, and NSI-MS Analysis—Protein bands were excised from gels and digested with trypsin as described (51, 52). The peptides were separated by nano-HPLC (LC Packings), introduced through a nanospray ionization source to an ion-trap mass spectrometer (LCQ-Deca, ThermoFinnigan) and analyzed by tandem mass spectrometry, as described (51, 52). Default score values used as cut-off parameters during TurboSequest searches were as follows, Xcorr > 1.0, ΔCn > 0.1, Snp > 500, and Rsp < 10, and the peptide mass tolerance was 1.0.

RNA Transcription and Gel-mobility Shift—The 32P-labeled RTE and CTE RNAs (34) were prepared as in a previous study (53), and unlabeled RNAs were synthesized using MEGAScript-T7 (Ambion, Austin, TX). 10 nM cold RTE or CTE RNA was added to 10 fmol of radiolabeled RTE or CTE RNA, respectively, to keep the RNA concentration constant for the different probes. Binding reactions were performed in 10 μl of RBB–250 in the presence of 2 μg of tRNA. After 30 min at room temperature, 1 μl of a solution containing 0.2 mg/ml heparin and 0.05% bromphenol blue was added to the reactions, following 10 min at room temperature. Samples were loaded onto a 6% (19:1 acrylamide:bisacrylamide ratio) non-denaturing polyacrylamide gel containing 50 mM Tris-Borate, pH 8.8, 0.5 mM
EDTA. Electrophoresis was carried out at 4 °C, and complexes were visualized by autoradiography.

**Cell Culture, Transfection, and Microscopy**—Human 293 and 293T cells were transfected using calcium phosphate technique. Human HeLa-derived HLTat cells were transfected with SuperFect (Qiagen). For indirect immunofluorescence, the fixed cells (54) were incubated with murine anti-HA antibody (Covance) at a 1:1000 dilution in phosphate-buffered saline in the presence of 0.2% bovine serum albumin, followed by Alexa 594-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR). For the shuttling assay, transfected HeLa cells were mixed with an excess of untransfected cells, and, after pretreatment with cycloheximide, the cells were fused using polyethylene glycol (54). For cotransfection experiments, reporter plasmids were used at 1 μg, in the absence or presence of 0.5 μg of plasmids expressing export factors. All transfections contained 0.2 μg of GFP (pFRED143 or pFRED25) or SEAP expression plasmids serving as internal control for efficiency of transfection and poly-A RNA preparation. Two days later, Gag (HIV p24gag antigen capture assay, Zepptomix), SEAP (Phospa-light kit, Applied Biosystems), CAT, and GFP fluorescence (54) were measured. Nuclear and cytoplasmic mRNA was prepared (25).

For RNA interference experiments, 2 × 10⁵ HeLa cells were transfected with 10 mm SMART pool siRNA (Dharmacon) targeted to RBM15 (GGACAGAGGTGAGCAGAT, GAAGT-AGGAAGCTGTGTAT, GGACACCCACCTATTATA, and GGTTGATAGTTGGGATATA) or non-targeting siRNA control (Dharmacon) using HiPerFect (Qiagen). One day later, the cells were re-transfected with the reporter plasmids using SuperFect (Qiagen). Culture media were collected 48 h later, and Gag and SEAP were measured. To control for the efficiency of RBM15 knockdowns, the cells were transfected with an RBM15-FLAG-expressing plasmid on day 1 and then transfected with either RBM15 siRNA pools or siRNA control pool on day 2. Cells were harvested in RBB-400 buffer on day 4, and the lysates were analyzed on immunoblots using murine anti-FLAG antibody (M2, Sigma) and horseradish peroxidase-conjugated anti-murine antibodies (Amer sham Biosciences). RNA interference pool-treated untransfected cells were analyzed using anti-RBM15 antiserum (ProteinTech Group, Inc., Chicago, IL) and anti-β actin antibody (Sigma). The proteins were visualized by enhanced chemiluminescence (ECL plus Western blotting Detection System, Amersham Biosciences) and autorography. Reverse transcrip tion-PCR of the endogenously expressed alternatively spliced RBM15 mRNAs was performed on total poly-A containing mRNAs isolated from HeLa and 293 cells using the Titan One Tube RT-PCR kit (Roche Applied Science), and the PCR products were sequenced.

**Recombinant Protein Expression and Protein Analysis**—Human RBM15 was produced in Escherichia coli BL21(DE3)pLysS (Novagen) from pGEX-6P-3-RBM15. Recombinant soluble GST-RBM15 protein was isolated after freezing the bacterial pellets in phosphate-buffered saline supplemented with 160 mm NaCl and protease inhibitor (PI, Roche Applied Science) at −70 °C for 30 min. The lysates were treated with DNase I (Roche Applied Science) and cleared by centrifugation. Glutathione-agarose beads (Roche Applied Science) were added, and the mixture was rotated for 1 h at 4 °C. The beads were washed three times, and the recombinant protein was eluted using a standard glutathione-containing buffer. Protein concentrations were estimated on Coomassie Blue-stained SDS-polyacrylamide gels.

**RNA Export from Xenopus Oocyte Nuclei**—The preparation of capped RTE and CTE-containing adenovirus precursor RNA, U1ΔSm RNA, and U6ΔSS RNA, unlabeled RTE, CTE, and CTEm36 RNAs and oocyte nuclei microinjections were described previously (34, 55, 56). RNA was extracted from a pool of five oocytes after proteinase K digestion, and equivalents of one-half oocyte were analyzed on 10% polyacrylamide gels containing 7 M urea.

**In Vitro Protein Binding Assays**—Metabolically labeled reticulocyte-produced proteins were synthesized in a coupled transcription/translation system (TNT T7 Coupled Reticulocyte Lysate System, Promega) and used in binding reactions cont-
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A

\[ \text{Cotransfection of RBM15: } - + - + \]

\[ \text{Fold activation by RBM15: } 1 6 \]

\[ \text{RNA export element: none RTE} \]

B

\[ \text{Cotransfection of RBM15: } - + - + - + - + - + \]

\[ \text{Fold activation by RBM15: } 1.4 27 4.5 1.8 1.7 \]

\[ \text{RNA export element: none RTE CTE RRE RRE+Rev} \]

C

\[ \text{Cotransfection of RBM15: } - + - + - + \]

\[ \text{Fold activation by RBM15: } 1.8 32 5.3 \]

\[ \text{RNA export element: none RTE CTE} \]

D

| RTE mutants | Fold activation of gag reporter mRNA | Additional fold activation by cotransfected RBM15 |
|-------------|-------------------------------------|-----------------------------------------------|
| RTE         | 10                                  | 27                                            |
| RTEm20      | 9                                   | 30                                            |
| RTEm21      | 14                                  | 20                                            |
| RTEm24      | 3                                   | 6                                             |
| RTEm25      | 1                                   | 1.7                                           |
| RTEm26      | 20                                  | 15                                            |
| RTEm27      | 1                                   | 1                                             |
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RESULTS

Identification of RBM15 as an RTE Binding Factor—Immo-
obilized, biotinylated RTE RNA was used to identify putative
binding factors from HeLa nuclear extracts in pull-down ex-
periments. CTE RNA was included as control in a parallel ex-
periment (Fig. 1A). These binding conditions enabled the specific
pull-down of NXF1 with CTE RNA but not with inactive
mutant CTEm36 RNA, lacking the NXF1 binding sites (3, 30),
confirming the binding specificity (data not shown). Four can-
didate RTE-specific binding factors were identified by microse-
quencing (Fig. 1A), which are RNA helicase A (SwissProt accession
number: O70133), RBM15 (Q96T37), heterogeneous
nuclear ribonucleoprotein G (P38159), and U1 small nuclear
ribonucleoprotein A (P09012). An additional band at ~100 kDa
could not be identified because of contamination with bovine
serum albumin and was not further studied. Subsequent exper-
iments did not support specific interactions of RNA helicase A,
heterogeneous nuclear ribonucleoprotein G, and U1 small
nuclear ribonucleoprotein A with RTE RNA (data not shown).
In contrast, RBM15 showed specific and functional interaction
with RTE as detailed below. RBM15 belongs to the spen (split
end) protein family and is conserved in eukaryotes from
Caenorhabditis elegans to humans (57), but its function had not
been investigated previously. Characteristic of this gene family
is the presence of three conserved RNA recognition motifs at
the N terminus and the SPOC (spen paralogous ortholog
terminal) domain at the C terminus.

Preferential Binding of RBM15 to RTE in Vitro—We
expressed recombinant RBM15 (isofrom AE+S, see Fig. 2A)
in bacteria and employed electrophoresis mobility shift assays to examine binding of RBM15 to RTE RNA (Fig. 1B).
Radiolabeled RTE (left panel) or CTE (right panel) RNAs were incubated with increasing amounts of bacterially pro-
duced GST-tagged RBM15. Approximately 50% binding of
RTE RNA was observed at 15 nM of RBM15 (left panel, lane
6), whereas no complex formation with CTE RNA was detectable (right panel, lanes 9–15), except for a weak band
detectable by using the highest concentration of RBM15
tested (50 nM, lane 16). These data show that recombinant
RBM15 binds directly to RTE RNA, in agreement with the finding from the pull-down assay (Fig. 1A), and demonstrate
that RBM15 interacts preferentially with RTE.

Identification of Novel Isoforms of RBM15—Ma et al. (48, 49)
reported three isoforms of RBM15 AE+S, S, and L, which share
aa 1–954 and have distinct C termini due to alternative splicing
the RBM15 mRNA (Fig. 2A). The isoform used throughout this
work is RBM15 AE+S spanning aa 1–977, which has also been
used by other investigators (48, 49, 58). An anti-RBM15 anti-
serum raised against the C terminus of RBM15 AE+S (aa 677–
977) became recently available, which detects all isoforms.
Testing human 293 and HeLa cells, we found several barely
visible bands of endogenous RBM15 migrating higher than the
major band, which migrates at ~100 kDa (Fig. 2B, lane 1). The
endogenous RBM15 is significantly smaller than our exog-
enously expressed RBM15 AE+S, which migrates at ~110 kDa
(lane 3). Upon inspection of the sequence, we noted that there
are two AUGs at residues 1 and 45, respectively. Our cDNA
expression plasmid contains the optimized Kozak AUG
sequence precluding initiation at downstream AUG. There-
fore, we generated an expression plasmid containing 900 nucleo-
tide of the authentic RBM15 5′-untranslated region obtained
from the cDNA clone. Interestingly, we found that two proteins
were produced (lane 2), one weaker band comigrating with the
RBM15 AE+S band shown in lane 3, and one strong, shorter
band, suggesting that the second AUG at position 45 is prefer-
entially used. This protein is slightly larger than the major
endogenous produced RBM15 (lane 1). Because the proteins

FIGURE 3. RBM15 stimulates RTE-dependent reporter gene expression. A, RBM15 activates CAT production from pDM138-RTE. CAT is only expressed from
the unspliced mRNA containing the cat gene embedded within the HIV-1-derived env intron. The RTE, the splice donor (SD) and splice acceptor (SA) sites are
indicated. HeLa cells were transfected in the absence (open bars) or presence (black bars) of RBM15 expressing vector. A representative experiment is shown.
The presence of RTE in pDM138-RTE promoted increased levels (~7-fold) of CAT expression compared with the parent pDM138, as expected (34). B, RBM15
activates RTE-dependent Gag expression. Gag is only expressed from the unspliced mRNA containing CTE, RTE, or RRE. HeLa cells were transfected with the
pNLgag plasmids carrying the indicated RNA export elements in the absence (open bars) or presence (black bars) of RBM15. pNLgagRRE was cotransfected with
0.1 μg of HIV-1 Rev expression plasmid. Mean Gag values and standard deviations are shown. The presence of RTE, CTE, or RRE/Rev protein led to an increase
in Gag production (10-, 124-, and 76-fold, respectively) as expected from previous studies (30, 34, 36). The additional -fold activation by exogenous RBM15 is
shown. C, RBM15 activates expression from unspliced Gag-reporter mRNA. pNLGagA lacks splice sites and was previously shown to produce only unspliced
mRNA (36). Transfection of the pNLGag or pNLGag containing RTE or the CTE in the absence or presence of the RBM15 expression vector was performed as
described for B. The cell extracts were analyzed for Gag expression as above. D, activity of RTE mutants correlates with the ability to respond to cotransfected
RBM15. The different RTE mutants shown were reported previously (35): RTEm27 (loop deletion; open box); m20, m25, and m26 (nucleotide changes, light gray boxes); and m21 and m24 (compensatory nucleotide changes, dark gray boxes). The RTE activity was measured as -fold activation when inserted into pNLgag
compared with the parent plasmid, not containing RTE, and were reported by Smulevitch et al. (35). -- and + signs next to the mutants indicate their activity.
HeLa cells were transfected with pNLgag containing the different RTE mutants in the absence or presence of RBM15. The additional -fold activation is shown.
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produced in lanes 2 and 3 are based on the isoform AE+S, they represent the longest isoforms. These data suggest that isoforms with different C termini (see Fig. 2A) are preferentially produced. Using semi-quantitative reverse transcription-PCR, we confirmed the presence of all three forms of alternatively spliced RBM15 mRNAs in 293 cells and the presence of the 5'-untranslated region. We then generated cDNA expression plasmids for all isoforms utilizing either the AUG initiator codon at residues 1 or 45 as outlined in Fig. 2A. In Fig. 2C, the major endogenous form of RBM15 comigrates with RBM15-L 45–957 initiated at the internal AUG #2. In subsequent experiments (data not shown), we confirmed that all isoforms function and localize to the nucleus like RBM15 AE+S, which was the isoform used for all our studies and is referred to as RBM15 in this work.

RBM15 Promotes Expression of RTE-containing Reporter mRNAs—It has been shown that RTE promotes nucleocytoplasmic transport of unspliced retroviral mRNA (34, 35, 59). We tested the hypothesis that RBM15 binding is important for RTE function. Two reporter plasmids, pDM138 (37), producing chloramphenicol acetyl transferase (CAT, Fig. 3A), and pNLgag (36), producing HIV-1 Gag (Fig. 3B), were used to test RBM15 function in cotransfection experiments in the presence or absence of exogenous RBM15. CAT or Gag are only produced from the unspliced mRNA transcripts, which require the presence of a strong RNA transport element such as CTE or RTE in cis (30, 34, 35, 59) or the Rev-responsive element RRE and HIV-1 Rev (36, 37, 61), as also shown in Fig. 3 (A and B, open bars). The presence of cotransfected RBM15 (black bars) further increased CAT expression by 6-fold from plasmid DM138-RTE but did not affect expression from pDM138 lacking RTE (Fig. 3A). Cotransfection of RBM15 also increased expression of gag from pNLGag containing RTE (27-fold), when compared with the expression obtained by the same plasmid in the absence of exogenous RBM15 (Fig. 3B). RBM15 also activated the RTE-containing pNLcagRTE (36) to similar extent like pNLgagRTE (Fig. 3C). Plasmid pNLcagRTE lacks the splice donor site located 5' to gag and produces only unspliced gag mRNA. These data indicate that RBM15 acts independent of splicing.

Different control gag plasmids were tested to study the specificity of RBM15 activation. No RBM15-induced stimulation was observed using the parent plasmid without RTE (Fig. 3, A–C), or the RTE-containing pNLgagRRE in the absence or presence of Rev (Fig. 3B), supporting specific action of RBM15 on RTE-containing mRNAs. Interestingly, a gag RNA containing the CTE transport element was reproducibly activated by RBM15 (4.5-fold, Fig. 3B). Activation of CTE-containing transcripts suggested that, although RBM15 acts preferentially on the RTE-containing RNA, it may have an additional general role in mRNA metabolism and may participate in NXF1-mediated export (see below).

RBM15 Function Requires the Presence of an Active RTE—A series of characterized RTE mutants (35), previously tested for their ability to activate gag expression (Fig. 3D), was examined in cotransfection experiments with the RBM15 expressing vector. The mutants used and their ability to induce Gag expression from pNLgag are shown in Fig. 3D. Cotransfection of RBM15 stimulated only the active RTE mutants m20, m21 m24, and m26. RBM15 did not activate the inactive mutants m25 and m27. Thus, the ability of exogenous RBM15 to further activate RTE-containing mRNA correlated with the activity of RTE. The data presented in Figs. 1 and 3 demonstrate that RBM15 specifically recognizes RTE RNA both in vitro and in vivo and that exogenous RBM15 promotes increased expression of RTE-containing mRNAs.

FIGURE 4. Knockdown of RBM15 by RNA interference preferentially inhibits RTE activity. A, RBM15 targeting siRNA pool specifically reduced RBM15 expression. HeLa cells were transfected with 0.5 µg of RBM15-FLAG expression plasmid together with 0.2 µg of plasmids expressing GFP and SEAP. The next day, the cells were transfected with 10 nM of a siRNA oligonucleotide pool specific to RBM15, or with nonspecific control siRNA. RBM15 expression was analyzed by Western immunoblot. GFP and SEAP were measured in cell lysates and supernatant, respectively. B, untransfected HeLa cells were treated with siRNA pool specific to RBM15 or with nonspecific control siRNA oligonucleotide pool for 2 days. Cell extracts were analyzed on Western immunoblot using anti-RBM15 antiserum (upper panel) and anti-actin antibody (lower panel). C, HeLa cells were transfected with 10 nM of pools of RBM15-specific or control siRNA oligonucleotides. One day later, the cells were retransfected with the pNLgag plasmid containing RTE(IAP92L23) or CTE together with a plasmid expressing SEAP. Gag expression was measured from the culture supernatant 2 days later. Gag and SEAP expression in the presence of control siRNA was normalized to 100%. This experiment was performed in quadruplicate plates; means and standard deviations are shown.
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Functional Knockdown of RBM15 by siRNA Inhibits RTE Function—Because we found that the exogenously expressed RBM15 stimulated the mRNA expression via RTE, we further investigated the effects of RBM15 depletion, performing functional knockdowns with siRNA. We first tested whether the selected pool of siRNAs was able to reduce cotransfected RBM15 levels. Transfection of a pool of four siRNAs targeting RBM15 led to a significant reduction of cotransfected FLAG-tagged RBM15 protein levels by ~75% (Fig. 4A). No off-target effects were observed on the expression of coexpressed GFP and SEAP transcripts, confirming the specificity of RBM15 knockdowns. Using an RBM15-specific antiserum, we further confirmed that the endogenous levels of RBM15 were also specifically and efficiently down-regulated by siRNA (Fig. 4B).

We next cotransfected HeLa cells with gag expression vectors containing either RTE or CTE together with a pool of siRNAs targeting the endogenous RBM15 or a pool of non-targeting siRNA. The production of secreted Gag protein was measured in the culture supernatants. Fig. 4C shows that RBM15-targeted siRNA significantly (by 74%) inhibited RTE-mediated gag expression. We also noticed an inhibitory effect, although to a lesser extent, on the expression of the CTE-containing gag mRNA (by 55%). This effect was expected, because we had observed (see Fig. 3B) that exogenous RBM15 activated CTE-containing gag mRNA expression. Cotransfection of SEAP plasmid, revealed only a small effect of the RBM15-specific siRNA on the expression of the SEAP transcript (~6% inhibition). These data provide another line of evidence that endogenous RBM15 is involved in RTE function. In addition, these findings further support our hypothesis that RBM15 participates in the NXF1 pathway, as evidenced by its effects on CTE-containing gag mRNAs.

RBM15 Directly Stimulates Nuclear Export of mRNA—We examined whether RBM15 is able to stimulate mRNA export directly by tethering RBM15 to a CAT reporter mRNA that is normally retained in the nucleus (25, 39). The tethering assay is based on the interaction of the RNA-binding N-terminal domain of λ phage antiterminator protein N (N) with its RNA-binding motif (boxB) (39, 62). CAT plasmids containing the boxB RNA binding elements (pDM128/B, Fig. 5A) or lacking the element (pDM128/PL) were cotransfected with plasmids expressing the factors of interest that were fused to the N-protein fusions to the full-length RBM15, or to the regions spanning aa 1–530 and 530–977, respectively, were visualized by indirect immunofluorescence using the anti-HA antibody. 4,6-Diamidino-2-phenylindole staining was performed to visualize nuclei.

Using this tethering assay, we verified (Fig. 5C) that cotransfection of plasmids, producing the N-peptide fusion to known mRNA export factors such as HIV-1 Rev and NXF1, promoted CTE-containing gag mRNA expression. Cotransfection of SEAP plasmid, revealed only a small effect of the RBM15-specific siRNA on the expression of the SEAP transcript (~6% inhibition). These data provide another line of evidence that endogenous RBM15 is involved in RTE function. In addition, these findings further support our hypothesis that RBM15 participates in the NXF1 pathway, as evidenced by its effects on CTE-containing gag mRNAs.

The cytoplasmic to nuclear ratio of the unspliced CAT mRNA is calculated. The stimulation of unspliced CAT mRNA export was normalized to that the level obtained in the absence of coexpressed N fusion-tagged proteins. Similar data were obtained in several independent experiments.
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A. pmol of competitor:

| Ad-RTE | RTE | 0.6 | 1.2 |
|--------|-----|-----|-----|
| T C N  | T C N| T C N| T C N|

- pre-mRNA (RTE)
- U1ΔSm
- spliced
- *
- U6Δss

B. pmol of competitor:

| Ad-RTE | CTE | 0.08 | 0.04 |
|--------|-----|------|------|
| C N C N| C N C N| C N C N|

- pre-mRNA (RTE)
- U1ΔSm
- spliced
- *
- U6Δss

C. pmol of competitor:

| Ad-CTE | RTE | 0.2 | 0.6 | 0.02 | 0.06 |
|--------|-----|-----|-----|-----|-----|
| C N C N| C N C N| C N C N|

- pre-mRNA (CTE)
- U1ΔSm
- spliced
- *
- U6Δss

D. 

| Ad pre-mRNA | Ad-RTE |
|-------------|--------|
| splicing    | RTE    |
| Export:     | yes    |

- intron-lariat
- spliced

- intron-lariat
- spliced

- yes

- yes
the N-RBM15 fusion protein also strongly promoted CAT expression (Fig. 5C). The export function of RBM15 depended on binding to the mRNA via the boxB elements, because it did not activate expression of the parent DM128/PL cat mRNA, lacking these elements (open bars). No increase in CAT expression from pDM128/B was found upon cotransfection of the RBM15 expression plasmid lacking the N-peptide (data not shown). Taken together, these data demonstrate that the observed stimulation by N-RBM15 (Fig. 5C) required direct interaction with the cat reporter mRNA. Testing the N- and C-terminal portions (RBM15 aa 1–530 and 530–977, respectively), both localizing to the nucleus (Fig. 5B), revealed that the export activity of RBM15 lies entirely within its C-terminal portion (Fig. 5C).

To verify that RBM15 acts to increase the nuclear export of cat mRNA, we analyzed the effects of RBM15 tethering on nucleocytoplasmic distribution of boxB-containing cat transcripts by Northern blots (Fig. 5D). As expected, we found that, in the absence of tethered export factors, the unspliced cat mRNA was retained in the nucleus, whereas the spliced transcript was efficiently exported to the cytoplasm (Fig. 5D), in agreement with Zolotukhin et al. (25). Thus, the ratio of unspliced (U, CAT-producing) to spliced (S, non-protein producing) cat mRNA in the cytoplasm can be used as a quantitative measure of export efficiency for unspliced cat transcript. The ratios of unspliced to spliced cat mRNA in the nuclear fractions were not affected by any of the N fusion export factors, confirming that these proteins act specifically at the nuclear export step. We found that tethering of Rev, NXF1, or RBM15 led to an increase in the ratio of unspliced to spliced mRNAs in the cytoplasm. This is in agreement with the reported properties of Rev and NXF1, the export factors for HIV RRE- and CTE-containing mRNAs, respectively, which increase the steady-state levels of cytoplasmic unspliced HIV mRNAs (29–33, 36, 63, 64). Together, these data provide direct evidence that RBM15 is a bona fide mRNA export factor with an effector domain located in its C-terminal portion. 

RTE Utilizes the NXF1 Pathway for Nuclear Export in Xenopus Oocytes—To study the mechanism of RTE-mediated nuclear export, we employed an in vivo RNA export competition assay using Xenopus laevis oocyte nuclei microinjected with radiolabeled adenovirus-derived pre-mRNA in the presence of increasing amounts of unlabeled competitor RNA (Fig. 6). Adenovirus-derived intron-lariats are efficiently exported from the nucleus (N) to the cytoplasm (C) only if they contain an active RNA export element such as RTE (34, 35) or CTE (56, 65), whereas the export of the spliced mRNA is not affected, as outlined in Fig. 6D. Coinjection of U1ÅSm RNA and U6Åss RNA served as quality controls and demonstrated proper function of the nuclear export machinery and intactness of the nuclei, respectively. This assay was previously used to show that export of the CTE-containing lariats utilizes the NXF1 pathway (3).

Using RTE RNA as competitor, we found interference with the nuclear export of the RTE-lariat at \(~0.6\) pmol of competitor (Fig. 6A, lane 3 versus lane 2 compared with lane 6 versus 5), indicating that the RTE export pathway is saturable. The saturating dose (~0.6 pmol of RTE RNA) was comparable to those previously reported for the nuclear export pathways utilized by U1 small nuclear ribonucleoprotein (0.5 pmol) and mRNA (0.1 pmol) (55). The RTE competitor did not affect the export of U1ÅSm RNA (representative of U1 small nuclear ribonucleoprotein export pathway). At 1.2 pmol of RTE competitor, we found some interference with splicing, resulting in reduced levels of intron-lariat and increased pre-mRNA levels (lane 1 compared with lane 7). Interestingly, the RTE competitor also strongly inhibited the export of spliced mRNA (see also Fig. 6C), because it was previously observed using the CTE as competitor in a similar assay (56). Thus, RTE is exported via a saturable pathway that overlaps with that of mRNA.

We next asked whether NXF1 was required for RTE-lariat export, despite the lack of a high affinity binding of NXF1 to RTE RNA (34). RTE RNA serves as a tool to inhibit NXF1 function, because NXF1 activity is specifically out-competed upon coinjection of very low amounts of CTE RNA (3, 56, 65). We found that the export of RTE-lariat was strongly inhibited by CTE competitor RNA, even at very low doses (Fig. 6B, lanes 2 versus 1 compared with lanes 6 and 5 or lanes 4 and 3). Excess RTE competitor had no effect on the export of U1ÅSm RNA, as expected (56). As an additional control, we used the mutant CTEm36, which lacks the high affinity NXF1-binding sites, but maintains the stems and the overall RNA structure, and does not compete for CTE export (3, 30). CTE and CTEm36 RNAs allow distinguishing NXF1 effects from other potential interactors (i.e. RNA helicase A (66–68)). Fig. 6B (right panel) shows that CTEm36 RNA competitor did not affect the RTE-lariat export (lanes 8 versus 7 compared with lanes 10 versus 9). These results demonstrate a role of NXF1 in the RTE-mediated nuclear export and suggest a potential interaction of RBM15 and NXF1.

In the converse experiment, we tested whether RTE RNA could compete for the export of the CTE-containing intron-lariat. Fig. 6C shows that coinjection of excess RTE RNA, using doses sufficient to compete RTE-lariat export (see Fig. 6A), had no effect on the export of the CTE-lariat (lanes 2 and 1 compared with lanes 6 and 5). Thus, RTE does not interfere with CTE export, which is in agreement with its low affinity to NXF1, as revealed by our in vitro binding studies (34). In contrast, the CTE-lariat export could be out-competed efficiently using even low doses of RTE competitor (~0.06 pmol, Fig. 6C,
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lanes 2 versus 1 compared with lanes 10 versus 9), as expected (56). These data show that CTE RNA competes for the export of both the CTE-lariat (Fig. 6C) as well as the RTE-lariat (Fig. 6B, left panel) with similar efficiency. These data are consistent with an essential role of NXF1 in RTE function.

RBM15 and NXF1 Bind to Each Other and Act Cooperatively—To test whether RBM15 and NXF1 can interact, we examined whether GST-tagged RBM15 protein can bind to reticulocyte produced radiolabeled proteins using an in vitro pull-down assay (Fig. 7A). We tested for interactions of RBM15 with the human NXF1, and as negative controls, with luciferase or with UAP56, a DExD/H box helicase involved in splicing and mRNA export (20, 46, 69, 70). NXF1, luciferase, and UAP56 were used at the same molar concentrations in the binding reactions. We found that the human NXF1 bound to RBM15 in vitro, whereas no interactions with luciferase, UAP56 or “empty” GST beads were observed. Similarly, we found that the mouse NXF2, a highly related mRNA export factor (27), interacted with RBM15 (data not shown). The binding assays were performed in the presence of RNase A, demonstrating that the identified interactions of RBM15 with the NXF proteins are RNA-independent.

We further analyzed the RBM15-NXF1 interaction in vivo using FLAG-tagged RBM15 protein and HA-tagged NXF1 in cotransfection experiments (Fig. 7B). Cotransfection of the HA-tagged UAP56, a nuclear protein that does not bind RBM15 in vitro, served as specificity control in the assay. Western blot analysis confirmed expression of HA-tagged proteins and similar levels of the FLAG-tagged RBM15. Coimmunoprecipitations using anti-FLAG antiserum confirmed the presence of HA-tagged NXF1, but not UAP56, in the RBM15-containing complex. RNase treatment of the cell extract did not affect this association, demonstrating RNA-independent interactions. Thus, both the in vitro and in vivo experiments confirmed the interaction between NXF1 and RBM15.

We then examined the RBM15-NXF1 interaction in more detail upon cotransfection of a series of FLAG-tagged RBM15 deletion mutants and HA-tagged NXF1 (Fig. 7C). The use of RBM15 deletion mutants identified the NXF1-interacting region within aa 530–977, whereas aa 1–530 and 530–750 did not associate. We concluded that the C-terminal portion of RBM15 contains an interaction site for NXF1 (Fig. 7C), as well as the signals necessary to promote RNA export, as revealed by the tethering assay shown in Fig. 5. Confirming the specificity of these assays, we used Western blot analysis to verify expression of HA-tagged NXF1 and of the FLAG-tagged RBM15 proteins, whereas coimmunoprecipitations using anti-FLAG antiserum verified the presence of HA-tagged NXF1 in the complex containing the intact RBM15 (1–977). Taken together, the in vitro and the in vivo data indicate a direct interaction between NXF1 and RBM15.

Cooperativity between RBM15 and NXF1—To probe the functional interaction of RBM15 and NXF1, we examined whether coexpression of RBM15 and NXF1 affects expression of RTE-containing gag mRNA. Human 293T cells were transfected with the gag reporter plasmid containing the RTE in the absence or presence of exogenous NXF1-p15, RBM15, or a combination of both factors. Fig. 8A shows that Gag expression...
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was increased in the presence of exogenous RBM15, as expected (see also Fig. 3), as well as, by exogenous NXF1-p15, although to a lesser extent. Importantly, we found that coexpression of both factors led to a further increase of Gag production, demonstrating cooperativity between RBM15 and NXF1. Addition of both factors had a more than additive effect on Gag expression (Fig. 4B). We found that cotransfection of pNLgagCTE with NXF1-p15 leads to elevation of Gag levels, as expected (71). Interestingly, the presence of exogenous RBM15 also activated CTE-mediated expression. These findings are in agreement with our previous observations, where RTE and CTE, present in close proximity on reporter mRNAs (see also Fig. 8), were found to cooperate in increased expression of RTE, as well as CTE-containing reporter mRNAs (Figs. 3 and 8), supporting a role of RBM15 in the NXF1 export pathway. Studies of mRNA export of retroviruses and retroelements have led to the identification and characterization of molecular steps important for understanding cellular gene expression. In this report, we show that RBM15 selectively binds to RTE RNA, a retrotransposon-derived transport element, with high affinity. RBM15 also specifically binds to NXF1, a key export receptor for cellular mRNAs. These data are consistent with a model where RBM15 links the RTE-containing mRNA to the NXF1 export pathway (Fig. 8C). Using a previously reported in vivo export assay (39), we demonstrate that RBM15 also increases the nucleocytoplasmic transport of reporter mRNAs containing the boxB RNA element when tethered to the boxB-binding λ phage N-peptide (Fig. 5), similar to the export factors HIV Rev and NXF1.

FIGURE 8. RBM15 and NXF1 act cooperatively. A and B, human 293T cells were transfected with 0.5 μg of pNLgagRTE (A) or pNLgagCTE (B) either alone or together with 0.5 μg of NXF1 and 0.1 μg of p15/NXT1 or 0.5 μg of RBM15 expressing plasmids alone or a combination. 0.1 μg of pStat, an HIV-1 Tat-expressing plasmid necessary to activate expression from the long terminal repeat promoter, was also included. Typical experiments performed in triplicates (A) or duplicates (B) are shown. The mean Gag measurements and standard deviations (A) or standard errors (B) are shown. For the transfections, the mean GFP values in panel A were 15,770, 20,971, 17,420, and 16,109 relative fluorescence units and in panel B were 25,902, 21,990, 24,972, and 17,694 relative fluorescence units, respectively. C, models for RBM15 participation in mRNA transport: RBM15 directly binds to the RTE RNA. NXF1 binds to the C-terminal portion of RBM15, and the NXF1-p15 heterodimer facilitates the signal for interaction with the NPC, thus RBM15 tethers the RTE-containing mRNAs to the NXF1 export pathway. Smulevitch et al. (59) reported that the presence of RTE and CTE on a reporter mRNA synergistically increased reporter gene expression. The presence of the two RNA binding sites in close proximity may facilitate efficient interaction of RBM15 and NXF1, resulting in increased reporter mRNA expression. RBM15 also acts as a NXF1 cofactor and, thereby, RBM15 interacts indirectly with CTE. Thus, the RBM15-NXF1 interaction with the RTE RNA or the CTE RNA allow for export and expression of the unspliced mRNAs. RBM15 is thought to interact via NXF1 and the components of EJC with the cellular mRNA, suggesting a role in general export of spliced mRNA.
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ously obtained for NXF1 using its high affinity target, CTE RNA, as competitor (56). One possible explanation is that RBM15 is involved in critical steps of mRNA export (see Fig. 8C). Our data suggest that RBM15 function on cellular mRNA is inhibited by the addition of excess of its high affinity binding RNA (RTE). Thus, these data are consistent with a role of RBM15 as NXF1 cofactor and provide evidence of a role of RBM15 in general mRNA export.

RBM15 belongs to a conserved family of proteins present as two members in most of the species examined, whereas there are three genes in humans and mice. The human family comprises the SPEN protein SHARP (SMRT/HDAC1-associated repressor protein); RBM15, also called One Twenty-Two (OTT); and the recently identified OTT3, also called RBM15b (72). SHARP has transcriptional repressor function (57, 73, 74), which is not shared by RBM15 or OTT3 (72). RBM15 is expressed in many tissues (49), but no function has been attributed to this protein. In this report, we demonstrate that RBM15 acts at the post-transcriptional level, particularly in mRNA export and expression. This function of RBM15 is clearly distinct from that reported for the related protein SHARP, which is involved in transcriptional suppression (57, 73, 74). In agreement with Hiriart et al. (72), we found no evidence that RBM15 acts as transcriptional repressor by testing the activity of different promoters such as HIV-1 long terminal repeat, SV40, or CMV (data not shown). Therefore, despite their evolutionary relationship, RBM15 and OTT3 appear to have functions distinct from that of SHARP. Interestingly, RBM15 has been found to fused to megakaryoblastic leukemia 1 protein (MKL1) in a translocation involving chromosome 1 and 22, resulting in acute megakaryoblastic leukemia (49, 58, 75–77). The fusion protein consisting of RBM15 at its N terminus and MKL1, a transcription factor (78) at its C terminus, could interact with the mRNA export machinery. Although the RBM15-MKL1 fusion protein was found to maintain the specific transactivator function of MKL1 (78), the fusion protein lost the post-transcriptional activating function of RBM15, as measured by its inability to activate RTE-mediated mRNA expression.3 However, it is possible that the RBM15-MKL1 fusion protein has transdominant suppressor function contributing to the oncogenic properties of RBM15-MKL1. Alternatively, the possible decrease of post-transcriptionally active RBM15 due to MKL1 fusion could affect mRNA regulation, potentially contributing to leukemogenesis. Elucidation of the role of RBM15 in mRNA export provides the basis for additional testable hypotheses on the oncogenic mechanism of RBM15-MKL1.

While this work was finalized, another member of the SPEN family, OTT3, was reported to bind to the Epstein-Barr virus early protein EB2. OTT3 was further shown to participate in splicing regulation of β-thalassemia mRNA, supporting its role in post-transcriptional steps of gene expression (72). Thus, both RBM15 and the related OTT3 participate in post-transcriptional control of gene expression. EB2 interacts with the SPOC domains of all three human SPEN family proteins SHARP, RBM15, and OTT3 (72). However, whereas SHARP and RBM15 interact with the C-terminal portion of EB2, OTT3 interacts with its N-terminal portion, demonstrating distinct properties despite the high level of homology in the SPOC domains (72). Interestingly, it is the C-terminal portion of RBM15 containing the SPOC domain that provides the interaction site with NXF1 as well as with the EB2 (72). Although there is no recognizable motif shared among these factors, a more in-depth analysis may reveal common structural requirements. Alternatively, distinct regions within the C-terminal portion of RBM15 interact with these factors. The function of OTT3 in RTE-mediated mRNA expression and its interaction with NXF1 are currently under investigation.

We propose that two structurally distinct but functionally analogous RNA export elements (RTE and CTE) have independently evolved (Fig. 8C). RTE, present in murine intracisternal A-particle retroelements (34, 35), emerged as high affinity ligand for RBM15. CTE, present in the related Type D simian retroviruses, has evolved to bind to NXF1 (29, 30, 33). It is believed that high affinity binding to export factors is essential to promote the export of the full-length unspliced mRNA of retroviruses or retroelements. In contrast, the vast majority of cellular mRNAs have to undergo splicing before export, resulting in a splicing-dependent deposition of export factors such as NXF1. In this report, we present evidence that RBM15 also has a role in the general mRNA export pathway, because RTE inhibits export of the spliced adenovirus mRNA from the Xenopus oocyte nuclei (Fig. 6). RBM15 also binds directly to the major export receptor for mRNAs, NXF1. We therefore propose that RBM15 plays a role in the general mRNA export pathway, by interacting with EJC via NXF1 (Fig. 8C). The molecular mechanism by which RBM15 participates in general mRNA metabolism remains the subject of further studies.

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Note Added in Proof—The isoform RBM15 L 45–957 is expressed from the RBM15 transcript variant L using the second AUG of the ORF and is named RBM15 L2 (GenBank under the accession number BK005915).

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