

# Translation Is Required to Remove Y14 from mRNAs in the Cytoplasm

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## Summary

**Background:** Y14 is an RNA binding protein which is part of a multiprotein complex, the exon-exon junction complex (EJC), that assembles on the exon-exon junctions of mRNAs produced by splicing. The position-specific binding of Y14 persists on mRNAs after their export to the cytoplasm. Thus, Y14, together with its interacting proteins, has the capacity to communicate to the cytoplasm the processing history of the mRNA, including the position of the removed introns, information that is likely to be important for defining premature termination codons. How Y14 and other components of the EJC are removed from mRNAs into the cytoplasm has not been determined.

**Results:** We show that Y14 but not another EJC component, Aly/REF, is present in polysome profile fractions containing one ribosome per mRNA. Using reporter constructs in an *in vitro* splicing/translation-coupled system, we show that Y14 remains associated with untranslated mRNAs but is removed from translationally active mRNAs. Importantly, mRNAs whose translation *in vivo* is prevented by the presence of strong secondary 5' UTR structure retain Y14 in the cytoplasm.

**Conclusions:** These findings indicate that Y14 remains associated with mRNAs in the cytoplasm until they are translated, and translation is required to remove Y14 from mRNAs. Thus, the process of translation removes the splicing-dependent EJC protein imprints, which most likely function in the surveillance of mRNAs to define premature termination codons and possibly also in modulating the translation activity of cytoplasmic mRNAs.

## Introduction

Although transcription and splicing can be uncoupled *in vitro*, pre-mRNAs are predominantly spliced cotranscriptionally *in vivo* [1]. Nascent transcripts become associated with hnRNP proteins and components of the splicing machinery including snRNPs. The hnRNP proteins that bind to chromatin-associated pre-mRNAs participate in various nuclear events, such as transcription regulation, alternative splicing, and 3' end processing [2, 3]. Whereas snRNPs dissociate from spliced mRNAs, many of the hnRNP proteins remain associated with spliced mRNAs in the nucleus, and the majority of these also accompany mRNAs to the cytoplasm, suggesting a role for these proteins in mRNA export and cytoplasmic mRNA metabolism [4, 5]. Consistent with this, several

of the hnRNP proteins have been found to function in mRNA translation, stability, or mRNA localization in the cytoplasm [3, 6–10].

In addition to the proteins that bind pre-mRNAs cotranscriptionally or shortly thereafter, splicing both removes and recruits specific proteins to the mRNA. These recruited proteins, in the form of a multiprotein complex are assembled in a sequence-independent, position-specific manner, near (~20 nt upstream of) exon-exon junctions [11–15]. Several components of this complex, called the exon-exon junction complex (EJC), have been identified and include SRm160 [12, 16], DEK [17], RNPS1 [18], Aly/REF [19], Y14 [11], magoh [20], and Upf3 [21, 22] (Figure 1A). SRm160 is a splicing enhancer rich in serine-arginine (SR) repeats, which functions by interacting with snRNPs, and SR proteins [16]. RNPS1 is a splicing coactivator and contains an RNA binding motif (RBD) [18]. DEK may also be a component of the EJC. It copurifies with SR proteins and interacts with SRm160 *in vitro*, but its interactions are not exclusive to mRNAs, as it also binds pre-mRNAs [17].

In the nucleus, the EJC stimulates mRNA export [19, 23]. This activity may be mediated, at least in part, by the RNA binding protein Aly/REF, which binds the mRNA export factor TAP (Figure 1B). Mammalian Aly/REF can bind directly to both TAP and RNA at the same time [24], and microinjection of recombinant Aly/REF in *Xenopus* oocytes enhances export of spliced mRNAs [19]. Temperature-sensitive mutants of Yra1p, the *S. cerevisiae* homolog of Aly/REF, are impaired in poly(A) RNA export [25]. However, the nuclear export of both spliced and unspliced mRNAs was inhibited by injection of anti-Aly/REF antibodies, suggesting that this protein might also function in the nuclear export of intronless mRNAs [26].

The EJC is highly dynamic, and several of its proteins, Aly/REF, Y14, magoh, RNPS1, and Upf3, shuttle between the nucleus and cytoplasm [11, 19, 21, 22], while others, including SRm160, do not appear to shuttle [22]. Only Y14 and possibly magoh, RNPS1, and Upf3 remain associated with newly exported mRNAs in the cytoplasm (Figure 1C). Aly/REF dissociates from mRNAs during or immediately after export [27], and although Upf3 and RNPS1 were found in RNP complexes containing Y14, Aly/REF, and TAP [21, 22], these proteins may not persist on mRNAs in the cytoplasm [23].

The final removal of Y14 and of other putative components of the cytoplasmic EJC (cEJC) from mRNAs has been suggested but not previously demonstrated to be mediated by the translation machinery (Figure 1D). This suggestion has been made in an attempt to explain the requirement for both pre-mRNA splicing and translation in the recognition of premature stop codons (PTCs) and activation of the nonsense-mediated degradation of mRNAs that bear PTCs [28, 29]. Because Y14 remains on the mRNAs after export to the cytoplasm in the same position, near exon-exon junctions, it has the necessary attributes to serve as a mark that could communicate to the translation machinery in the cytoplasm information that is needed to distinguish between PTCs and legitimate termination codons. Here we show that Y14

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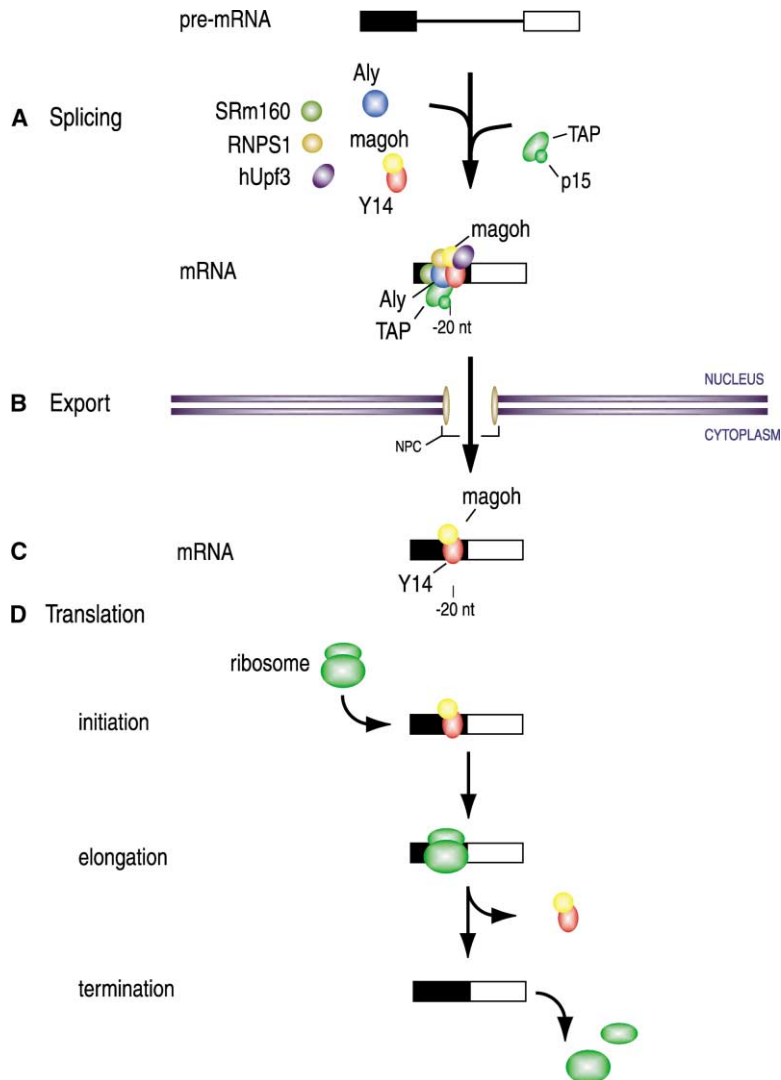


Figure 1. Schematic Representation of the Formation, Transport, and Removal of the EJC from mRNAs  
See text for details.

is found on mRNAs in the cytoplasm of somatic cells. In particular, Y14 is present in polysome profile fractions corresponding to mRNAs that are associated with one ribosome. We developed an assay to analyze the requirement for translation for the removal of Y14. We show both *in vitro* and *in vivo* that mRNA translation is required to remove Y14 from spliced cytoplasmic mRNAs. These findings support the conclusion that Y14, likely together with magoh, RNPS1, and Upf3, serves as the splicing-dependent marker that communicates to the translation machinery in the cytoplasm the information necessary for surveillance of PTCs and thus for activation of NMD.

## Results

### Y14 Cosediments with Cytoplasmic mRNAs Associated with Monosomes

Previous studies demonstrated that the splicing-dependent position-specific binding of Y14 on mRNAs exon-exon junctions persists after export to the cytoplasm in

*Xenopus* oocytes [11]. The splicing substrates used in previous experiments did not contain an open reading frame encompassing the exon-exon junctions, as is the case for most cellular mRNAs. Thus, it seemed possible that these substrates did not behave as natural mRNAs with regards to their interaction with the translation machinery. Consequently, it was not clear whether Y14 persists on mRNAs until they engage the translation machinery in the cytoplasm, and whether translation is required to remove Y14 from mRNAs. Furthermore, these studies were carried out in *Xenopus* oocytes, and similar information on somatic cells was thus not available.

To determine whether Y14 is present in fractions containing mRNAs associated with ribosomes in somatic cells, cytoplasmic extracts from HeLa cells were prepared and resolved by sedimentation on sucrose gradients. The RNA content in the collected fractions was analyzed by spectrophotometry to identify the position of ribosomal complexes [monosomes (80S) and polysomes; Figure 2A, upper panel]. The presence of ribo-

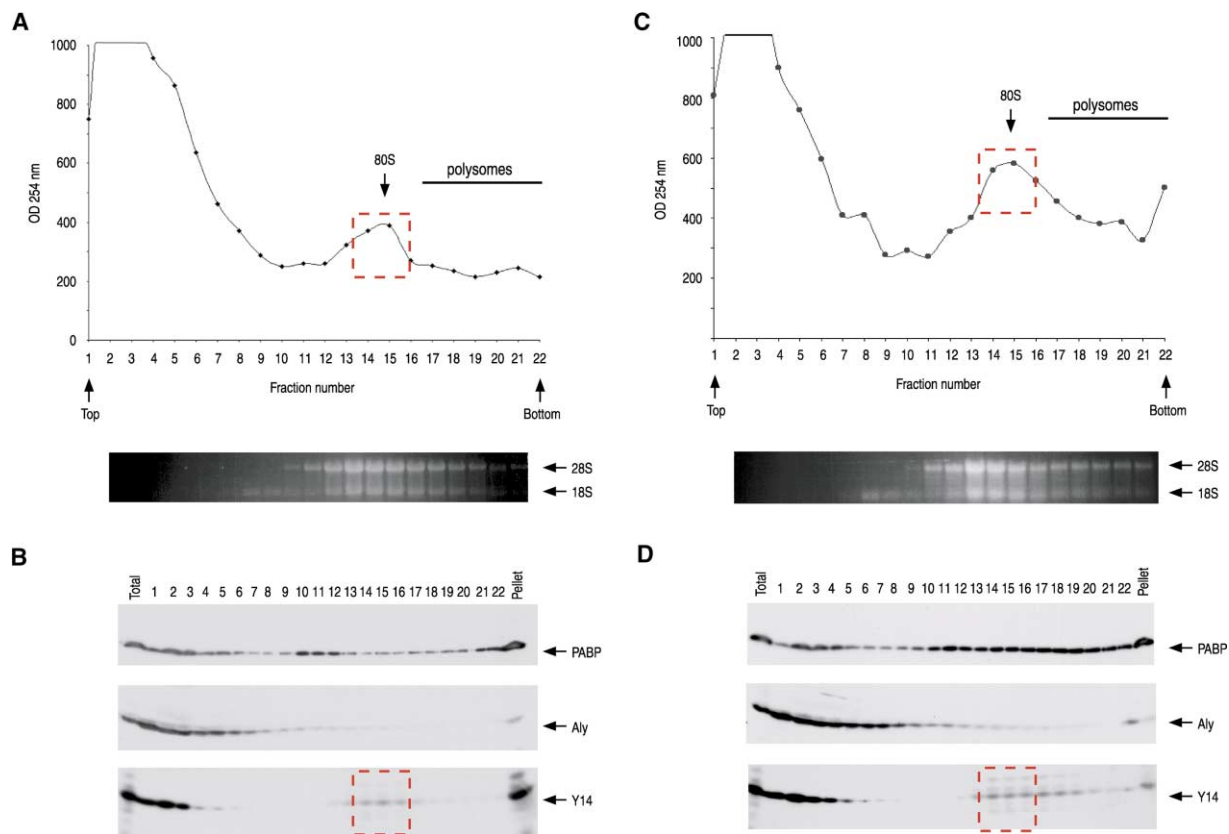


Figure 2. Endogenous Y14 Cosediments with mRNAs Associated with Monosomes

(A) (Upper panel) Optical density profile from 15%–40% sucrose gradient of cytoplasmic HeLa cell extract. x axis represents the fraction number, and y axis the optical density at 254 nm. Fraction numbers are from top (fraction 1) to bottom (fraction 22) of gradient. (Lower panel) Agarose gel analysis of fractions 2 to 21.

(B) Immunoblotting analysis of gradient fractions. Total proteins from each fraction were resolved by SDS-PAGE and analyzed by Western blotting with anti-PABP 10E10 (top), anti-Aly/REF 11G5 (middle), and anti-Y14 1F12 (bottom). This experiment was reproduced four times under various experimental conditions. Pellet represents sedimented proteins.

(C and D) Experiment was done as in (A) and (B), except that cells were first treated with puromycin (0.1 mg/ml) for 2 hr.

somal RNA was also verified by agarose gel electrophoresis (Figure 2A, lower panel). The proteins in the same fractions were resolved by SDS-PAGE and analyzed for the presence of Y14 and other components by immunoblotting with the indicated antibodies (Figure 2B). Figure 2B shows that most of the cytoplasmic Y14 and Aly/REF is present in lighter complexes at the top of the gradient. However, Y14 displayed a second peak in the 80S (monosome) region (Figure 2B; dashed box), which was sensitive to puromycin treatment of the cells, whereas the sedimentation profile of Aly/REF was not affected under these conditions (Figures 2C and 2D). Likewise, we found that cosedimentation of Y14 with 80S particles is sensitive to EDTA (data not shown). The presence of the poly(A) binding protein PABP in the gradient fractions was analyzed as control for the sedimentation profile to monitor the presence of mRNA. These results are in good agreement with previous reports indicating that Y14 but not Aly/REF remains stably bound to mRNAs in the cytoplasm [27] and suggests that Y14 interaction persists at least until the initial stages of association between the mRNA and the translation machinery.

### Translation Is Required to Remove Y14 from mRNAs In Vitro

To determine whether mRNA translation is required for the removal of bound Y14 from mRNAs, we developed an *in vitro* splicing/translation-coupled assay, using reporter constructs. The splicing substrate derived from the chicken  $\delta$ -crystallin pre-mRNA (CDC) and its corresponding mRNA has been extensively characterized *in vitro* and *in vivo* [11, 27, 30]. However, this substrate did not contain an open reading frame. We therefore modified the CDC pre-mRNA and mRNA to include an AUG that would serve as a translation initiation codon at the beginning of exon 1 and a flag-encoding sequence with a TGA termination codon at the end of exon 2 (pre-CDC and CDCm; Figure 3A).

To monitor the effect of translation on the association of Y14 with the modified CDC, a stable hairpin structure of estimated  $\Delta G = -30.3$  kcal/mole was inserted upstream of the AUG in exon 1 (5'-secpre-CDC and 5'-sec-CDCm; Figure 3A). This secondary structure was previously shown to efficiently inhibit mRNA translation both *in vitro* and *in vivo* [31]. Stable secondary structure insertion in the 5' UTR of the modified CDC constructs

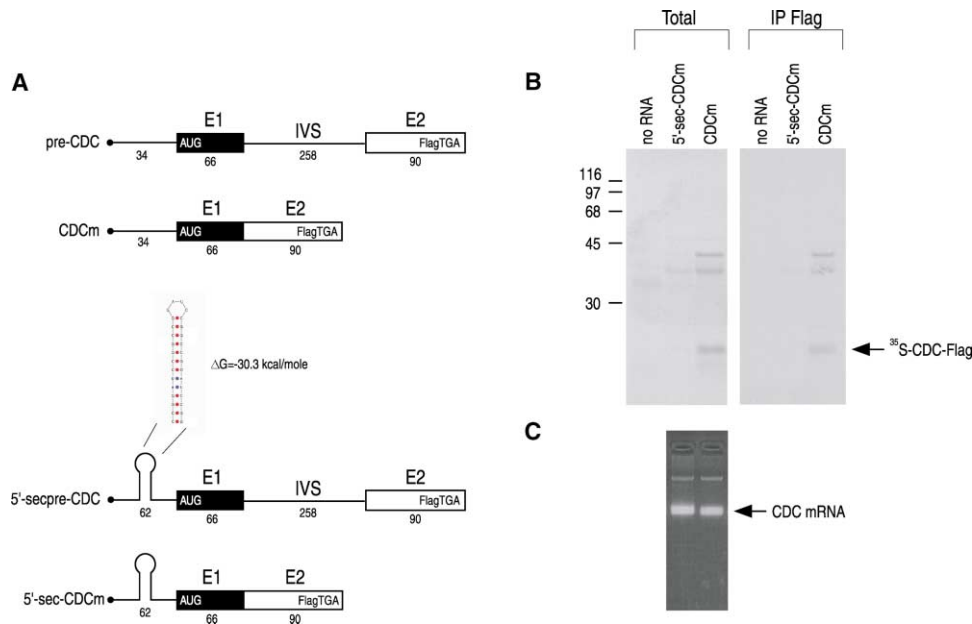


Figure 3. The Stable RNA Secondary Structure 5'-sec Inhibits Translation of CDCm when Placed in its 5' UTR

(A) Schematic representation of the novel CDC splicing substrates (pre-CDC and 5'-sepre-CDC) and corresponding intronless mRNAs (CDCm and 5'-sec-CDCm). Numbers below each construct represents sizes (nt) of 5' UTR, exons (E1 and E2), and intron (IVS). Position, predicted structure, and sequence of 5'-sec are shown in enlargement. Proposed 5'-sec secondary structure was predicted by the mFold algorithm [37, 38].

(B) In vitro translation of [ $^{35}$ S]methionine 5'-sec-CDCm and CDCm in rabbit reticulocyte lysate. Total (left) or anti-Flag immunoprecipitated (right) translation products were resolved by SDS-PAGE and revealed by autoradiography.

(C) Agarose gel analysis of capped 5'-sec-CDCm and CDCm input RNAs used in (B).

rather than general translation inhibitors was used to inhibit translation in order to achieve a more complete and reproducible block in protein synthesis. To verify that this stable secondary structure also efficiently inhibits translation of CDCm, equal amounts of capped 5'-sec-CDCm and CDCm mRNAs were incubated in rabbit reticulocyte lysates translation system containing [ $^{35}$ S]methionine (Figure 3C). Total (Figure 3B, left) and anti-Flag immunoprecipitated (right) protein products were resolved by SDS-PAGE and visualized by autoradiography. Figure 3B shows that protein was produced from CDCm but not from 5'-sec-CDCm, indicating that the secondary structure efficiently inhibits translation of 5'-sec-CDCm in vitro, and that the open reading frame introduced in CDCm is indeed translated.

Because modification of the CDC sequence might affect splicing efficiency of the substrates, the relative activity of 5'-sepre-CDC and pre-CDC was examined in vitro. The relative splicing efficiency of the substrates was found to be similar and comparable to the original CDC (data not shown). As changes in the CDC sequence might also affect assembly of exon-exon junction complexes, the interaction of Y14 with the novel splicing substrates was characterized. Aliquots from splicing reactions of capped 5'-sepre-CDC and pre-CDC were collected to verify splicing efficiency (Figure 4A, lanes 3 and 4). The remaining reaction volumes were used in immunoprecipitation experiments with anti-Y14 (4C4) and a nonimmune antibody (SP2/0) as a control (lanes

5 to 8). Total, immunoprecipitated, and unbound RNAs were extracted and resolved by electrophoresis on denaturing polyacrylamide gels. In agreement with previous studies, Figure 4A shows that Y14 was preferentially associated with spliced mRNAs (lanes 6 and 8) and importantly that Y14 bound equally well to 5'-sec-CDCm and CDCm produced by splicing (compare lanes 6 and 8). The interaction of Y14 with mRNAs was splicing dependent, as no significant amount of 5'-sec-CDCm or CDCm processed as above was immunoprecipitated with the anti-Y14 antibody (Figure 4B, lanes 5 to 8). Since these new splicing substrates are efficiently spliced, associate with Y14 in a splicing-dependent manner, and contain a regulated open reading frame which is recognized by the translation machinery, this system is suitable to analyze the role of the translation machinery in the removal of Y14 from mRNAs.

To determine whether translation is required to remove Y14 from spliced mRNAs, 5'-sepre-CDC and pre-CDC splicing reactions were incubated in rabbit reticulocyte lysates before immunoprecipitation. During this step, CDCm but not 5'-sec-CDCm will be translated, and if translation is required to remove Y14 from the spliced mRNAs, more 5'-sec-CDCm should be immunoprecipitated with the anti-Y14 (4C4) antibody. Figure 4C shows that after translation considerably more Y14 was associated with untranslated 5'-sec-CDCm as compared to CDCm. Thus, these results suggest that translation can remove Y14 from mRNAs in vitro.

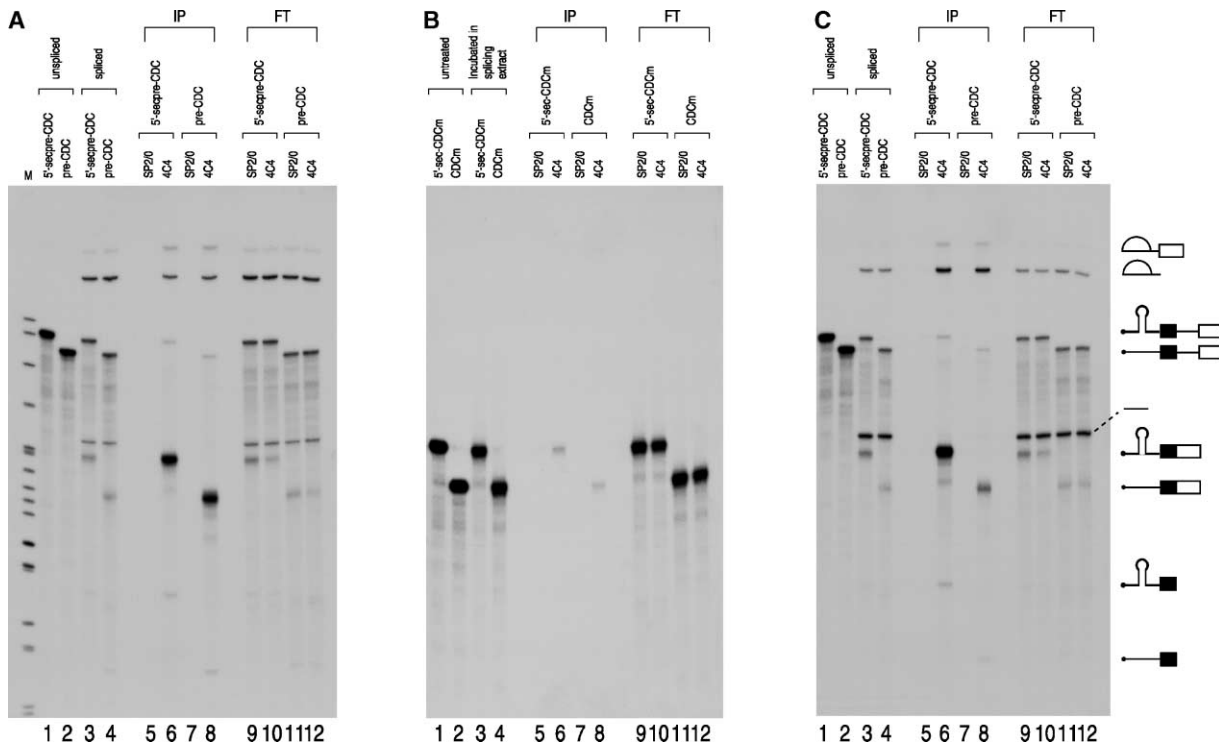


Figure 4. mRNA Translation Is Required to Remove Y14 from mRNAs In Vitro

(A) Characterization of the relative splicing efficiencies and association of Y14 on 5'-secpre-CDC and pre-CDC splicing substrates.  $^{32}\text{P}$ -labeled 5'-secpre-CDC or pre-CDC (unspliced; lanes 1 and 2) were incubated in HeLa nuclear splicing extracts (spliced inputs, lanes 3 and 4) and used in immunoprecipitation experiments with the indicated antibodies (IP, lanes 5 to 8).  
 (B) Y14 does not associate with intronless 5'-sec-CDCm and CDCm. Experiments were done as in (A), except that  $^{32}\text{P}$ -labeled intronless 5'-sec-CDCm and CDCm were used in this assay.  
 (C) Splicing reactions of  $^{32}\text{P}$ -labeled 5'-secpre-CDC or pre-CDC (unspliced; lanes 1 and 2) were incubated in rabbit reticulocyte lysates (spliced inputs, lanes 3 and 4) and used in immunoprecipitation experiments with the indicated antibodies (IP, lanes 5 to 8). Splicing products and intermediates are indicated on the right. Splicing input (lanes 3 and 4 of panels [A]–[C]) represents 10% of material used in immunoprecipitations, and lanes 9 to 12 represent 10% of the material recovered after immunoprecipitation (FT, flowthrough; M, molecular weight marker).

### Removal of Y14 from Cytoplasmic mRNAs In Vivo Requires Translation

The results presented in Figure 4 suggest that the binding of Y14 persists on mRNAs in the cytoplasm until the mRNAs are engaged by the translation machinery. However, in this system, spliced mRNAs are directly accessible to the protein synthesis machinery rather than first undergoing nuclear export through the nuclear pore complex. Consequently, it is possible that the composition of the post-export exon-exon junction complexes might be different from that of complexes produced by splicing in vitro. To address the possibility that translation is required to remove Y14 from mRNAs in vitro because it is more stably bound to transcripts in the presence of other complex components, the requirement of translation was analyzed in vivo. 293T cells were cotransfected with the intronless reporter construct Ad2m and either pcDNA3, 5'-secpre-CDC, or pre-CDC. Ad2m served as a control for transfection efficiency and for Y14 binding specificity. Transfected cells were treated with digitonin to prepare cytoplasmic lysates. Cytoplasmic extracts were used in immunoprecipitation experiments with the indicated antibodies, and bound RNAs were analyzed by RNase protection assay with RNA antisense probes. The antisense probe

used to detect spliced 5'-sec-CDCm and CDCm did not encompass the 5' untranslated region (5' UTR), and thus protected fragments from either constructs should be of the same size. Figure 5 shows that although comparable amounts of 5'-sec-CDCm and CDCm were generated (lanes 6 and 9), Y14 was associated almost exclusively with the translationally inactive 5'-sec-CDCm. The association of Y14 with 5'-sec-CDCm and CDCm was splicing dependent, since only low levels of Ad2m were immunoprecipitated with the anti-Y14 antibody (lanes 5, 8, and 11). Together, these results demonstrate that Y14 remains associated with mRNAs in the cytoplasm until the mRNAs engage the translation machinery and that cytoplasmic translation is required to remove Y14 from mRNA exon-exon junctions.

### Discussion

We show here that Y14 is present in polysome profile fractions corresponding to mRNAs that are associated with one ribosome in the cytoplasm of somatic cells. This suggests that Y14 remains bound to spliced mRNAs in the cytoplasm at least until the mRNAs engage the translation machinery. Previous experiments in *Xenopus* oocytes have shown that Y14 and its tightly associated

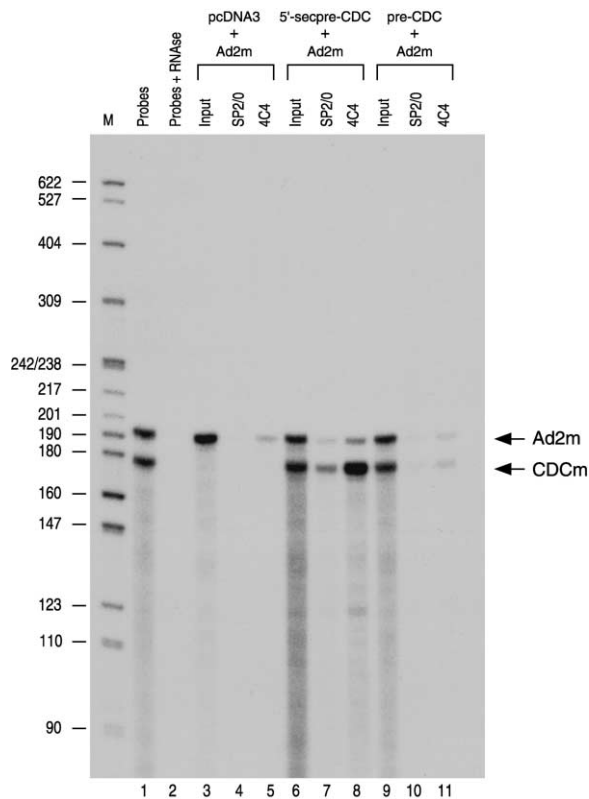


Figure 5. mRNA Translation Is Required to Remove Y14 from Cytoplasmic mRNAs In Vivo

Cytoplasmic extracts from HEK293T cells cotransfected with pAd2m and either pcDNA3 (lanes 3 to 5), 5'-secpre-CDC (lanes 6 to 8), or pre-CDC (lanes 9 to 11) were used in immunoprecipitation experiments with the indicated antibodies. Bound RNAs were extracted and analyzed by RNase protection assay with RNA antisense probes. Lane 1 represents 0.5% of input probes used in the assay. Protected fragment of Ad2m and CDCm are indicated on the right. Coimmunoprecipitated 5'-sec-CDCm (lane 8) and CDCm (lane 11) represents 60% and 4% of input, respectively. Lane M, molecular weight marker.

protein magoh assemble into the exon-exon junction complex (EJC) and are present on mRNAs after their export to the cytoplasm. This suggested that Y14, together with magoh, could provide the critical link between splicing and translation and thus function in nonsense-mediated mRNA decay (NMD). NMD is a process by which mRNAs containing mutations that cause premature termination codons (PTC) are selectively degraded [32]. A further link between Y14 and NMD was provided by the observation that Y14 interacts with the NMD factor Upf3 and that Upf3 is also part of the EJC [21]. Another EJC component, RNPS1, has been shown to interact with Upf3 [19] and thus is also likely to participate in mRNA surveillance. Upf3 is predominantly nuclear and thought to recruit Upf2 upon nuclear export [33]. Upf2 is also an essential factor in NMD and it interacts, in addition with Upf3, with the NMD factor Upf1, a protein associated with eukaryotic translation release factors, which is required to trigger NMD of PTC-containing mRNAs.

The presence of Y14 and possibly also magoh Upf3

and RNPS1 in ribosome-containing mRNPs is consistent with their role in providing the splicing-dependent imprints that distinguish PTCs from bona fide stop codons for the translation machinery in the cytoplasm. To explain the functional link between splicing and translation, we previously suggested that Y14 and more recently also magoh and Upf3 remain stably associated with spliced mRNAs in the cytoplasm at the exon-exon junctions, at least until the first round of translation [11, 20, 21]. However, the splicing substrates used in previous studies to address this issue did not contain open reading frames encompassing the exon-exon junction, and this issue could therefore not be addressed. It is possible that the stability of the association of Y14 with cytoplasmic mRNAs in *Xenopus* oocytes is due to the absence of an open reading frame in the splicing substrates used and consequently in the inability of these mRNAs to recruit and be translated by 80S ribosomal complexes (assembled ribosomes; for review, see [34]). Since injected mRNAs without open reading frames likely undergo translation initiation, the process by which the initiator AUG is recognized, Y14 complexes might only be removed from mRNAs by ribosomes during the elongation phase and not by the small (40S) ribosomal subunit during the initiation phase of protein synthesis. Interestingly, numerous mRNAs contain introns within their 5' UTRs. Thus, it will be important to determine whether the Y14 complex is assembled upstream of exon-exon junctions in the 5' UTR of these mRNAs and if the translation initiation process is sufficient to remove this complex from mRNAs.

We show here in vitro and in vivo that Y14 does not significantly associate with a translating mRNA produced by splicing but that it remains bound to a translationally silent mRNA in the cytoplasm. Thus, translation is required to remove Y14 from mRNAs in the cytoplasm. The observation that Y14 is bound to mRNAs associated with ribosomes also raises the possibility that it has a role in mRNA translation. Pre-mRNA splicing was previously shown to enhance mRNA translation activity [35]. Of the known cytoplasmic EJC components, Y14 and magoh are the best candidates to mediate this activity. It will be interesting to determine whether these proteins or other as yet unidentified cEJC components are responsible for this activity. The in vitro splicing/translation-coupled system should be a useful tool to characterize these functional aspects of the cEJC.

### Conclusions

We demonstrated that the EJC protein Y14 remains associated with mRNAs until mRNAs engage ribosomes in the cytoplasm. Using a novel in vitro splicing/translation-coupled system, we found that translation is required to remove Y14 from spliced mRNAs. We further showed that Y14 remains bound to translationally silent mRNAs but not to translated mRNAs in vivo.

### Experimental Procedures

#### Plasmids

Pre-CDC and CDCm were generated by polymerase chain reaction (PCR) using pCDC and pCDCm as templates, respectively [11]. Primers were designed to include an KpnI site and ATG or an XhoI site, Flag sequence, and TGA in the forward and reverse oligonucleo-

tides, respectively, to introduce an open reading frame in the reporter constructs. 5'-secpre-CDC and 5'-sec-CDCm were generated as described above, except that the following sequence was included in the forward primer: 5'-CCGGATCCGGCCGGATCCGGC CGGATCCGG-3'. PCR products were subcloned into pcDNA3 (Invitrogen), and sequences were verified by automated DNA cycle sequencing. The plasmid pAd2m was previously described [11].

#### Polysome Profile

HeLa S3 cells were grown in suspension in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; complete DMEM). Mid-log phase cells were collected by centrifugation at  $1000 \times g$  for 20 min at 4°C. Cell pellet was washed with cold phosphate-buffered saline (PBS) containing 100 µg/ml cycloheximide (Sigma) and with RSB 250 (10 mM Tris-HCl [pH 7.4], 2.5 mM magnesium chloride, 250 mM sodium chloride, 100 µg/ml cycloheximide). Cells were resuspended in one volume of RSB 250 containing 35 µg/ml digitonin (Calbiochem) and protease inhibitors (Complete™; Roche), incubated on ice for 5 min, and disrupted by gentle passage through a 25 gauge needle (4X). Extract was centrifuged at  $3000 \times g$  for 40 s at 4°C to pellet nuclei and debris, and cytoplasmic lysate was clarified by centrifugation at  $11,000 \times g$  for 15 min at 4°C. Supernatant was resolved on a 15%–40% sucrose gradient prepared in RSB 250 containing protease inhibitors. The sucrose gradient was centrifuged at 5°C in a Beckman SW41.1 rotor at  $18,000 \times g$  for 18 hr. Following centrifugation, fractions were collected from the top of the gradient with a BioComp fractionator. Fractions were analyzed by immunoblotting as described below.

#### Antibodies and Immunoblotting

Anti-Y14 antibodies, 1F12 (immunoblotting), and 4C4 (immunoprecipitation) were previously described [27]. Anti-PABP, 10E10, anti-Aly, 11G5 [27], and nonimmune mouse IgG, SP2/0 [11], were described elsewhere. Peroxidase-coupled anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories.

For immunoblotting, polypeptides were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.45 µm nitrocellulose membrane. Membranes were blocked in blotting solution (PBS-5% nonfat milk) for at least 30 min at room temperature (RT), rinsed with cold PBS, and incubated with primary antibodies in PBS-3% bovine serum albumin (BSA) for 1 hr at RT. Membranes were washed three times 10 min with PBS-0.1% NP-40, incubated with secondary antibodies for 45 min at RT, and washed four times with PBS-0.1% NP-40 prior to secondary antibody detection. Detection of peroxidase-coupled secondary antibodies was performed with an ECL Western blotting detection kit (Amersham Pharmacia Biotech, Inc.).

#### In Vitro Transcription and Splicing

To prepare in vitro transcription templates, 5'-secpre-CDC, pre-CDC, 5'-sec-CDCm, and CDCm were linearized with XhoI and agarose-gel purified (Qiagen). Nonradiolabeled mRNAs were generated with T7 polymerase, according to the manufacturer's instructions (Promega). In vitro splicing reactions, preparation of <sup>32</sup>P-UTP-RNAs, and of HeLa nuclear extracts was performed as previously described [36].

#### In Vitro Translation

Nonradiolabeled mRNAs were translated in reticulocyte lysates in the presence of [<sup>35</sup>S]methionine (Amersham Pharmacia Biotech, Inc.) according to the instructions provided by the manufacturer (Promega). For the splicing/translation-coupled assay, 0.1 volume of splicing reaction was added to the translation mixture (70% rabbit reticulocyte lysate, 1% -Leu amino acid mix, 1% -Met amino acid mix, and 0.9 units/µl RNasin). Samples were incubated for 1 hr at 30°C and processed for immunoprecipitation as described below.

#### Immunoprecipitation

Immunoprecipitation from splicing reactions was performed as follows: splicing reactions were diluted in 10 volumes of HNT buffer (10 mM Tris-HCl [pH 7.5], 150 mM sodium chloride, 0.05% Triton X-100, 0.9 units/µl RNasin, and protease inhibitors [Complete™; Roche]) and incubated with antibodies immobilized on protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Inc.) for 1 hr

at 4°C. Complexes were washed five times with HNT buffer and treated with proteinase K for 30 min at 37°C. RNAs were extracted with phenol/chloroform and analyzed on denaturing 6% polyacrylamide gels. Immunoprecipitation from splicing/translation-coupled samples was performed as above, except that samples were diluted with three volumes of HNT buffer prior incubation with antibodies.

#### Transient Transfections and Cell Extracts

HEK293 cells were plated on 100 mm dishes, and grown in complete DMEM. At 50% confluency, cells were transfected with plasmid DNA (2 µg) using Geneporter (GTS Technologies) according to the manufacturer's recommendations. At 24 hr posttransfection, cells were washed twice with cold PBS, collected by scraping, and centrifuged at  $1000 \times g$  for 15 min at 4°C. Cell pellets were resuspended in RSB 100 (10 mM Tris-HCl [pH 7.4], 2.5 mM magnesium chloride, 100 mM sodium chloride, 0.9 units/µl RNasin, and protease inhibitors [Complete™; Roche]) containing 35 µg/ml digitonin. Cells were incubated on ice for 5 min and disrupted by gentle passage through a 25 gauge needle (4X). Extracts were centrifuged at  $3000 \times g$  for 40 s at 4°C to pellet nuclei and debris, and cytoplasmic lysates were clarified by centrifugation at  $11,000 \times g$  for 15 min at 4°C. Immunoprecipitations and RNA extractions were performed as described above.

#### RNase Protection Assay

To prepare in vitro transcription templates, CDCm and pAd2m were linearized with KpnI and HindIII, respectively. Linearized plasmids were agarose-gel purified (Qiagen), and antisense mRNAs were generated with SP6 polymerase, according to the manufacturer's instructions (Promega). Preparation of <sup>32</sup>P-UTP-RNA probes was performed as previously described [36]. Extracted RNAs from transiently transfected HEK293T cells were assayed by RNase protection assay with radiolabeled probes as described by the manufacturer (RPA II kit; Ambion). Protected fragments were analyzed on denaturing 6% polyacrylamide gels.

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