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Human mRNA Export Machinery Recruited to the 5′ End of mRNA

Hong Cheng,[1] Kobina Dufu,[1] Chung-Sheng Lee,[1] Jeanne L. Hsu,[1] Anusha Dias,[1] and Robin Reed[1,*]
[1] Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
*Contact: rreed@hms.harvard.edu
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SUMMARY

Pre-mRNAs undergo splicing to remove introns, and the spliced mRNA is exported to the cytoplasm for translation. Here we investigated the mechanism for recruitment of the conserved mRNA export machinery (TREX complex) to mRNA. We show that the human TREX complex is recruited to a region near the 5′ end of mRNA, with the TREX component Aly bound closest to the 5′ cap. Both TREX recruitment and mRNA export require the cap, and these roles for the cap are splicing dependent. CBP80, which is bound to the cap, associates efficiently with TREX, and Aly mediates this interaction. Together, these data indicate that the CBP80-Aly interaction results in recruitment of TREX to the 5′ end of mRNA, where it functions in mRNA export. As a consequence, the mRNA would be exported in a 5′ to 3′ direction through the nuclear pore, as observed in early electron micrographs of giant Balbiani ring mRNPs.

INTRODUCTION

During expression of protein-coding genes, nascent pre-mRNA transcripts undergo extensive processing in the nucleus and are then exported to the cytoplasm for translation. These processing steps, which are thought to occur cotranscriptionally, include capping at the 5′ end, splicing to remove introns, and polyadenylation at the 3′ end (for reviews, see Bentley, 2002, 2005; Hirose and Manley, 2000). The integrity of the mRNA is also monitored by quality-control mechanisms such as nonsense-mediated decay (NMD), which eliminates mRNAs with premature termination codons (Maquat, 2000; Peltz et al., 1993). Each of the steps in gene expression is carried out by distinct multicomponent machines that are both physically and functionally coupled to one another (for reviews, see Bentley, 2002, 2005; Hirose and Manley, 2000). A hallmark of metazoan genes is the presence of numerous introns. There are often as many as 50 in a single gene with lengths of thousands of nucleotides. In contrast, introns are not present in most yeast genes, and those genes that do contain them usually have one small intron. In keeping with this observation, coupling gene expression steps to splicing is more prevalent in metazoans, whereas coupling gene expression steps to transcription is more common in yeast (for reviews, see Reed, 2003; Reed and Cheng, 2005).

The conserved TREX complex provides one of the best examples of the different mechanisms for coupling in yeast versus metazoans, as this complex is associated with the transcription machinery in yeast and the splicing machinery in humans (for review, see Reed and Cheng, 2005). The human TREX complex contains the proteins UAP56 and Aly (seen as Sub2 and Yra1 in yeast) as well as the multisubunit THO complex (Jimeno et al., 2002; Masuda et al., 2005; Strasser et al., 2002). In yeast, Sub2 and Yra1 are cotranscriptionally recruited to nascent transcripts by the THO complex (Abruzzi et al., 2004; Strasser et al., 2002; Zenklusen et al., 2002). In contrast, the human TREX complex is loaded onto mRNA during splicing (Masuda et al., 2005). RNA interference and biochemical studies in metazoans and genetic analyses in yeast indicate that the conserved TREX complex functions in mRNA export (Gatfield et al., 2001; Herold et al., 2003; Luo et al., 2001; Rehwinkel et al., 2004; Strasser et al., 2002; Zhou et al., 2000; Jimeno et al., 2002; Le Hir et al., 2001; Rodrigues et al., 2001; Strasser and Hurt, 2001; Stutz et al., 2000).

In previous work, UAP56 and Aly were identified as components of the exon junction complex (EJC) (Gatfield et al., 2001; Le Hir et al., 2000a, 2001). This complex binds ~20 nts upstream of the exon-exon junction of spliced mRNAs (Le Hir et al., 2000a). The EJC was also reported to contain the mRNA export receptor TAP/p15 (Le Hir et al., 2001; Tange et al., 2004), as well as factors such as eIF4A3, Upf3, RNPS1, and Y14 that function in NMD (Chan et al., 2004; Ferraiuolo et al., 2004; Gatfield et al., 2003; Gehring et al., 2003; Kim et al., 2001a, 2001b; Lykke-Andersen et al., 2001; Palacios et al., 2004; Shibuya et al., 2004; Singh and Lykke-Andersen, 2003). Thus, in the current model for mRNA export in humans, the NMD and mRNA export machineries are "deposited" on the mRNA during splicing and are bound at every exon-exon junction as components of the EJC (for review, see Tange et al., 2004).

In this study, we sought to determine whether the human THO complex is also deposited on the mRNA in

EJCs. Unexpectedly, this analysis led to a new model for export of mRNAs in metazoans, revealing that the human TREX complex is recruited in a splicing- and cap-dependent manner to a region near the 5′ end of the mRNA. In the new model, recruitment of TREX complex to mRNA occurs via an interaction between the TREX component Aly and the cap-binding protein CBP80. In contrast, the EJC is recruited to every exon-exon junction independently of the 5′ cap/CBP80 and does not contain the mRNA export machinery.

RESULTS

TREX Is Bound Near the 5′ End of mRNA

UAP56 and Aly associate with the THO complex to form TREX (Masuda et al., 2005). The human THO complex, which contains the proteins hTho2, hHpr1, fSAP79, fSAP35, and fSAP24, exists as a stable complex in the nucleus and in nuclear extracts (Chan et al., 2004; Masuda et al., 2005). In light of previous reports that UAP56 and Aly are components of the EJC (Gatfield et al., 2001; Le Hir et al., 2000a, 2001), we asked whether the THO complex is also present in the EJC. To investigate this possibility, we used the same strategy previously used to define EJC components (Le Hir et al., 2000a, 2001). Specifically, we spliced a pre-mRNA in vitro and used oligonucleotide-directed RNase H to cleave the mRNA at specific sites (Figure 1A; Le Hir et al., 2000a, 2001). The digested RNAs were then immunoprecipitated with an antibody to the THO complex. Initially, we used an hTho2 antibody that coimmunoprecipitates the five THO proteins in a tight, stoichiometric complex (Masuda et al., 2005; see also Figure 5B). As controls, we used antibodies to Aly and the NMD protein eIF4A3 (Chan et al., 2004; Ferraiuolo et al., 2004; Kim et al., 2001b; Le Hir et al., 2000a, 2001; Shibuya et al., 2004).

As expected, full-length spliced Ftz mRNA was immmunoprecipitated by antibodies to eIF4A3, Aly, UAP56, and hTho2 but not by the negative control antibody (Figure 1B, lanes 1–6; Chan et al., 2004; Ferraiuolo et al., 2004; Le Hir et al., 2000a, 2001; Masuda et al., 2005; Shibuya et al., 2004). When Ftz mRNA was cleaved 60 or 168 nucleotides upstream of the exon-exon junction, the mRNA fragment containing the EJC was immunoprecipitated by eIF4A3 (Figure 1B, lanes 9 and 15). Unexpectedly, however, different results were obtained with the Aly antibody. The CAP fragment rather than the EJC fragment was efficiently immunoprecipitated when the mRNA was cleaved 60 or 168 nts upstream of the exon junction (Figure 1B, lanes 10 and 16). These data indicate that Aly does not bind to mRNA at the EJC but instead binds upstream of it.

To determine where on Ftz mRNA the THO complex binds, we carried out the same immunoprecipitations using the hTho2 antibody. When the mRNA was cleaved at −60 nts, the THO complex was mainly detected on the CAP fragment (Figure 1B, lane 12). When the mRNA was cleaved closer to the 5′end of the mRNA at −168 nts,

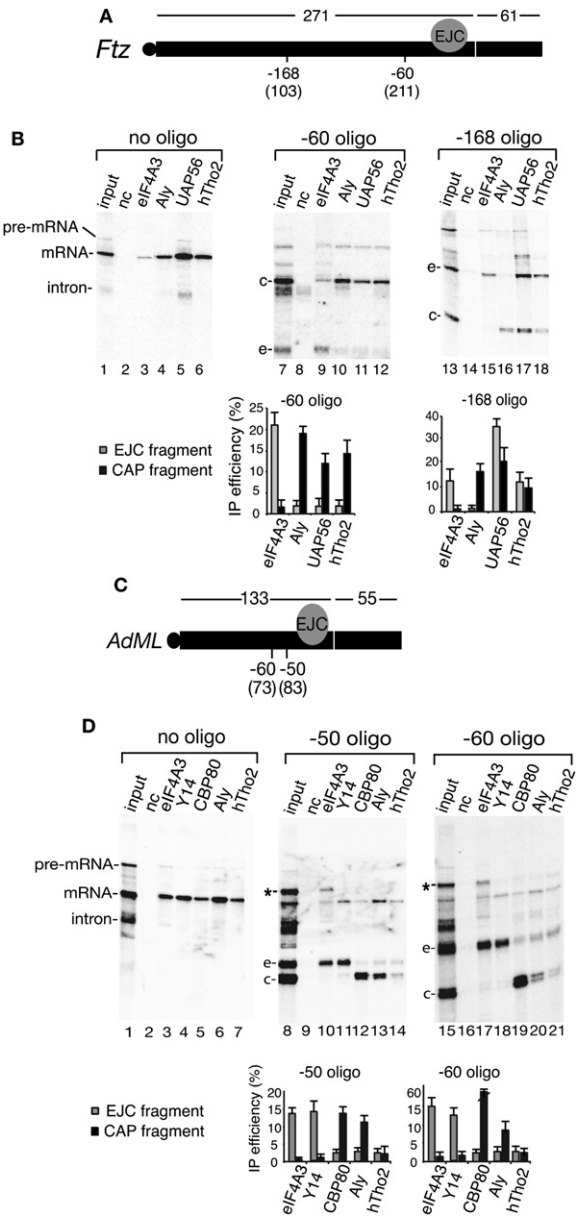

Figure 1. TREX Binds to mRNA Upstream of the EJC

(A and C) Schematic of Ftz and AdML mRNAs. The numbers indicate the RNase H cleavage sites and are designated relative to the exon junction "−" or cap "(."

(B) The total splicing reaction (lane 1) or reactions after RNase H cleavage (lanes 7 and 13) were immunoprecipitated with the indicated antibodies. One-fourth of the input was loaded. The negative control antibody is indicated by nc. The RNA fragments containing the EJC and the 5′ cap are indicated by e and c, respectively. Quantitation of three independent experiments is shown. Error bars represent standard deviations (n = 3).

(D) Same as (B), except with AdML RNA. The asterisks indicate RNA fragments from cleaved pre-mRNA. Quantitation of three independent experiments is shown. Error bars represent standard deviations (n = 3).

the THO complex was detected more on the EJC fragment (Figure 1B, lane 18). Considering that the THO complex is large and multicomponent, it is likely to be bound to a larger portion of the mRNA than an individual protein. We next investigated the binding site of the TREX component UAP56. Significantly, as observed with the THO complex and Aly, the binding site of UAP56 is distinct from that of the EJC (Figure 1B). UAP56 was detected on the CAP fragment when the mRNA was cleaved at −60 nts and on both the CAP and EJC fragments at −168 nts (Figure 1B, lanes 11 and 17). These data indicate that Aly binds to the mRNA closest to the cap and THO complex and that UAP56 binds downstream of Aly but upstream of the EJC.

In previous studies, an AdML RNA substrate was used to conclude that the export machinery is at the EJC (Le Hir et al., 2000a). Thus, we next used this same AdML RNA to determine whether substrate-specific differences could account for the discrepancy between our work and the previous studies (Figure 1C). The cap-binding complex (CBC), which is a heterodimer of CBP20 and CBP80, binds to the 5′ cap of RNAP II transcripts (Izaurralde et al., 1992, 1994). Thus, we used an antibody to CBP80 as an additional control for the CAP fragment. An antibody to the EJC component Y14 was used as an additional control for the EJC fragment (Kim et al., 2001b). As shown in Figure 1D, this antibody, as well as antibodies to eIF4A3, CBP80, Aly, and hTho2, immunoprecipitates the spliced mRNA (lanes 1–7). When AdML mRNA was cleaved 50 or 60 nts upstream of the exon-exon junction, eIF4A3 and Y14 were detected on the EJC fragment as expected (Figure 1D, lanes 10 and 11). In striking contrast, the Aly antibody, like the CBP80 antibody, efficiently immunoprecipitated the CAP fragment when the mRNA was cleaved at −50 nts (Figure 1D, lanes 12 and 13). Aly was also detected more strongly on the CAP than the EJC fragment when the mRNA was cleaved at −60 nts (Figure 1D, lane 20). The cleaved mRNA was not efficiently immunoprecipitated with the hTho2 antibody, possibly because the large THO complex is bound near the cleavage sites. However, of the RNA that was immunoprecipitated, the THO complex was detected at similar levels on both the CAP and EJC fragments (Figure 1D, lanes 14 and 21). Together with the analysis of Ftz mRNA, the data indicate that TREX is not bound to the mRNA ~20 nts upstream of the exon-exon junction where the EJC is located but is instead bound upstream of it.

TREX Is Bound Near the 5′ End of mRNA, and an EJC Is Bound at Every Exon-Exon Junction

It has been proposed that an EJC is located at each exon-exon junction in the mRNA (Culbertson and Leeds, 2003; Le Hir et al., 2000b; Schell et al., 2002; Wagner and Lykke-Andersen, 2002). To test this possibility and to determine whether a TREX complex is bound upstream of the EJC on every exon, we examined Smad pre-mRNA, which contains two introns (Figure 2A). When this pre-mRNA was spliced, both introns were excised

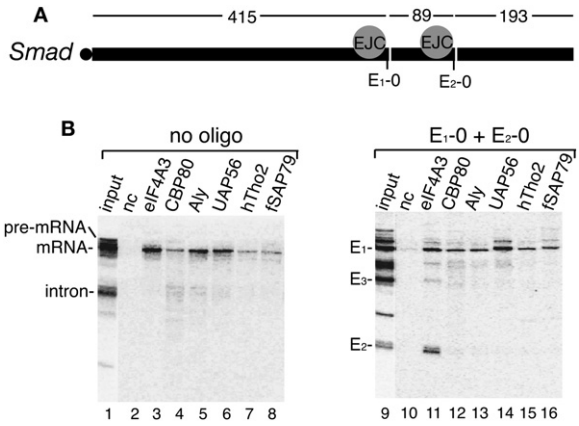

Figure 2. TREX Binds to Exon 1 of mRNA, and the EJC Binds at Exon-Exon Junctions

(A) Schematic of Smad mRNA showing RNase H cleavage sites.

(B) Splicing reactions after RNase H cleavage were used for immunoprecipitation with the indicated antibodies. E indicates exon.

(Figure 2B, lane 1). As expected, antibodies to eIF4A3, CBP80, and TREX components (Aly, UAP56, hTho2, and fSAP79) immunoprecipitated the intact Smad mRNA (Figure 2B, lanes 1–8). When Smad mRNA was cleaved at each exon-exon junction, the three exons were present on distinct RNA fragments (Figure 2B, lane 9). Significantly, eIF4A3 was detected on both exons 1 and 2, whereas CBP80 and all of the TREX components were only detected on exon 1 (Figure 2B, lanes 9–16). This observation indicates that an EJC is bound at each exon-exon junction, whereas TREX is bound only on exon 1.

Aly Is the TREX Component Bound Closest to the 5′ Cap

To further localize TREX components on exon 1, we used oligonucleotide-directed RNase H cleavage to digest Ftz and Smad mRNAs at sites located 12 nts apart along the entire length of the exon (data not shown). The oligonucleotides that both cleaved well and were appropriately located (based on Figure 1 and data not shown) were used to cleave the spliced mRNA, followed by immunoprecipitation with antibodies to Aly, hTho2, CBP80, or eIF4A3 (Figures S1A–S1F). This analysis revealed that CBP80, Aly, the THO complex, and the EJC all have binding sites that can be readily distinguished from one another (Figure S1). Specifically, Aly is the TREX component located closest to CBP80 on the mRNA, the THO complex is bound downstream of Aly, and the EJC is bound downstream of the THO complex (Figure S1). The positions where these factors bind vary between the different mRNAs, possibly because of differences in their exon 1 lengths and/or sequences.

Several reasons may account for the discrepancy between our work and the previous work concluding that Aly, UAP56, and Tap/p15 are EJC components. For example, in two of the key studies (Gatfield et al., 2001; Le Hir

et al., 2001), the RNA fragment that was used for the work extended from the 5′ cap all the way down to the exon junction, so that it was not actually possible to draw a conclusion regarding where the EJC binds. Consistent with our conclusion that TREX is not an EJC component, more recent reports now indicate that the mRNA export machinery is not a core component of the EJC (Ballut et al., 2005; Tange et al., 2005) or is not in the EJC at all (for review, see Moore, 2005). Our data are the first to demonstrate that TREX is bound near the 5′ end of the mRNA rather than at the EJC.

5′ Cap- and Splicing-Dependent Recruitment of TREX to mRNA

The observation that TREX binds to mRNA near the 5′ end raises the possibility that the 5′ cap plays a role in recruitment of TREX. To test this possibility, we affinity-purified spliced mRNPs containing or lacking the cap (Figure 3A). Western analysis of total protein in the purified mRNPs revealed that eIF4A3 is present at the same level in both the presence and absence of the cap (Figure 3B). In striking contrast, CBP80, Aly, and hTho2 are only detected in the capped, spliced mRNP (Figure 3B). Thus, the 5′ cap functions in recruitment of TREX, but not the EJC, to spliced mRNA.

Previous studies showed that efficient recruitment of TREX is also splicing dependent, and that TREX colocalizes with splicing factors in nuclear speckles in vivo (Masuda et al., 2005; Zhou et al., 2000). To confirm the splicing dependence of TREX recruitment, we affinity-purified mRNP complexes assembled on spliced mRNA or the identical cDNA transcript (Figure 3C). As shown in Figure 3D, western analysis revealed that CBP80 is present at the same level in both types of mRNP complexes. In contrast, TREX components (hHpr1, UAP56, and fSAP79) and the EJC protein eIF4A3 are abundant in the spliced mRNP but not in the cDNA transcript mRNP. We conclude that efficient recruitment of TREX to mRNA is both cap and splicing dependent.

The 5′ Cap Functions in Export of Spliced mRNA

Previous studies showed that efficient export of mRNA is splicing dependent (Luo and Reed, 1999). The observation that recruitment of TREX to mRNA is cap dependent as well as splicing dependent raises the possibility that the 5′ cap also functions in mRNA export. To test this possibility, we performed *Xenopus* oocyte microinjection export assays (Hamm and Mattaj, 1990) using AdML or Ftz pre-mRNAs containing or lacking the cap. Both capped and uncapped RNAs had similar stability and splicing kinetics (Figures 4A and 4B). In contrast, the export efficiency of the capped mRNAs was dramatically enhanced relative to the uncapped mRNA (Figures 4A and 4B). For AdML, the uncapped mRNA was not exported at all, and for Ftz, the mRNA export efficiency was increased by the presence of a cap (up to 10-fold). To confirm this result, we used tRNA export as an internal control for the efficiency of export in individual oocytes. These data

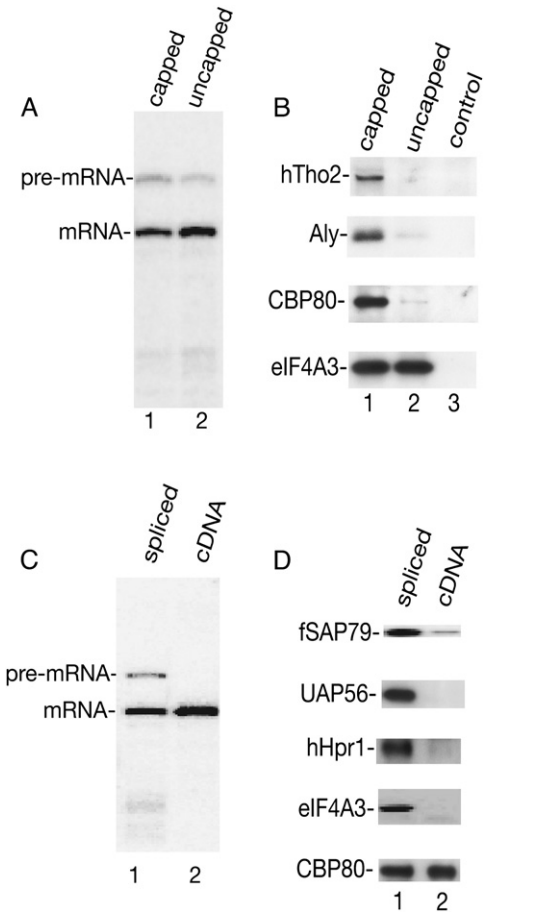

Figure 3. Recruitment of TREX to mRNA Is 5′ Cap and Splicing Dependent

(A) Capped (lane 1) or uncapped (lane 2) AdML spliced mRNPs were isolated from gel filtration fractions, and total RNA was separated on an 8% denaturing polyacrylamide gel.

(B) Total protein from equal amounts of MS2-MBP affinity-purified spliced mRNPs assembled on capped (lane 1) or uncapped (lane 2) AdML mRNA was separated on 4%–12% SDS-PAGE, and western analysis was performed. Control (lane 3) is total protein isolated from a parallel MS2-MBP affinity-purification using AdML mRNA without MS2-binding sites.

(C) Total RNA isolated from gel filtration fractions containing spliced mRNP (lane 1) or cDNA-transcript mRNP (lane 2) was separated on an 8% denaturing polyacrylamide gel.

(D) Total protein from equal amounts of MS2-MBP affinity-purified AdML mRNPs was separated on 4%–12% SDS-PAGE, and western analysis was performed. Spliced mRNP (lane 1) and cDNA-transcript mRNP (lane 2) are shown.

revealed that tRNA was exported with the same efficiency in all oocytes, whereas the capped, but not the uncapped, AdML mRNA was efficiently exported (Figure 4C). We conclude that the 5′ cap is required for efficient export of spliced mRNA.

As efficient TREX recruitment is not only cap dependent but also splicing dependent, we next asked whether the role of the cap in mRNA export is also splicing dependent. Accordingly, AdML or Ftz cDNA transcripts containing or

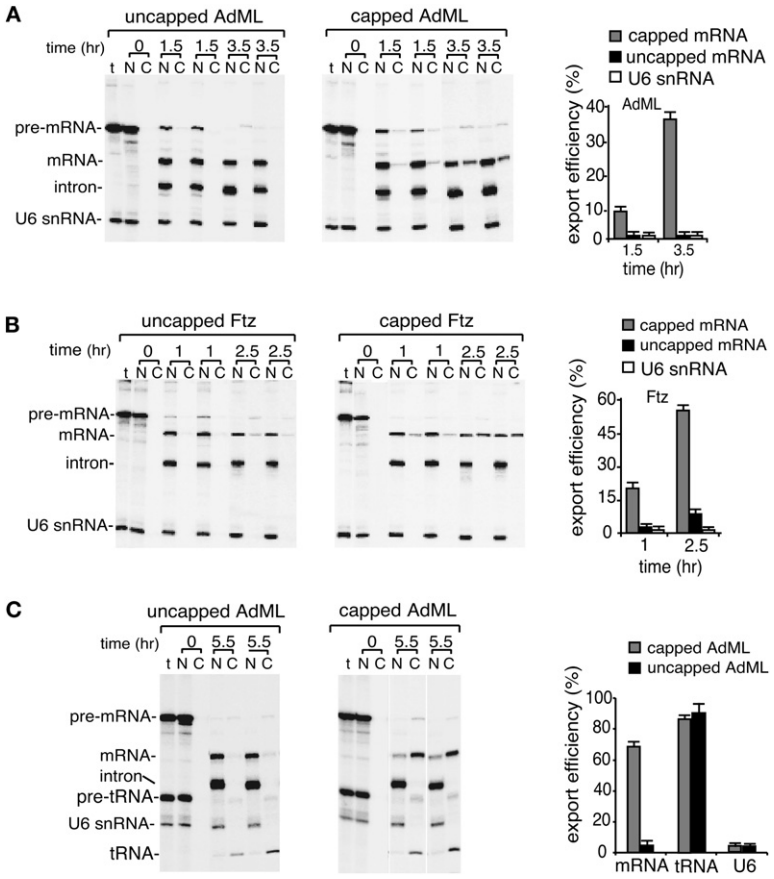

Figure 4. Export of Spliced mRNA Is Cap Dependent

(A) Equal amounts of capped or uncapped AdML pre-mRNA were mixed with U6 snRNA and microinjected into *Xenopus* oocyte nuclei (lane t). After incubation at 18°C for the times indicated, oocytes were dissected, and the distribution of RNA in the nucleus (N) and cytoplasm (C) was analyzed on an 8% denaturing polyacrylamide gel. The export efficiency, defined by calculating the amount of mRNA in the cytoplasm relative to the total mRNA at the earliest time point when the spliced mRNA is detected (the 1.5 hr time point for AdML and 1 hr time point for Ftz), is shown in the graph. Quantitation of three independent experiments is shown. Error bars represent standard deviations (n = 3).

(B) Same as (A), except Ftz pre-mRNA was used. Quantitation of three independent experiments is shown. Error bars represent standard deviations (n = 3).

(C) Same as (A), except pre-tRNA, which is processed to tRNA and exported, was added to the microinjection mixture. Quantitation of three independent experiments is shown. Error bars represent standard deviations (n = 3).

lacking the cap were microinjected into *Xenopus* oocyte nuclei. As shown in Figure S2, these RNAs were degraded and/or inefficiently exported. Moreover, the low level of export that did occur is not significantly different in the presence or absence of the cap (Figure S2). The same results were obtained with RNAs of random sequence (Figure S2). Thus, in striking contrast to spliced mRNAs, the cap does not have a role in export of cDNA transcripts or random RNAs. This observation confirms previous studies in which the cap and CBC played only a minimal or no role in export of cDNA transcripts (Izaurralde et al., 1995; Jarmolowski et al., 1994; Lewis and Tollervey, 2000; Masuyama et al., 2004).

TREX Interacts with CBC via Protein-Protein Interactions

Considering that the cap functions in recruiting TREX, we next asked whether CBC interacts with TREX. As the 5′ cap is not involved in recruitment of the EJC component eIF4A3 (Figure 3), we also asked whether CBC interacts with eIF4A3. To ask these questions, we carried out immunoprecipitations from RNase-treated nuclear extract followed by western blots. Antibodies to the CBC component CBP80, TREX components (Aly, UAP56, hTho2, and fSAP79), eIF4A3, and the translation factor eIF3b (as a negative control) were used for this analysis. As shown in Figure 5A, antibodies to eIF4A3 and eIF3b efficiently immunoprecipitated their respective proteins but not each other, CBP80, or any of the TREX components (Figure 5A, lanes 1 and 2). Significantly, however, CBP80 antibodies not only immunoprecipitated CBP80 but also coimmunoprecipitated Aly, UAP56, hTho2, and fSAP79 (Figure 5A, lane 3). Reciprocally, CBP80 was present in the Aly, UAP56, hTho2, and fSAP79 immunoprecipitates (Figure 5A, lanes 4–7). We conclude that TREX, but not the EJC, interacts with CBC via protein-protein interactions.

As another approach for determining whether CBC associates with TREX, we analyzed the total proteins present in CBP80 and hTho2 immunoprecipitates on a Coomassie-stained gel followed by mass spectrometry. As shown in Figure 5B (lane 1), the TREX components were readily detected among the CBP80-associated proteins. In contrast, no EJC components were detected. These data indicate that TREX, but not the EJC, is a predominant factor that associates with CBC.

CBP80 and Aly Associate via Protein-Protein Interactions

To further investigate the interaction between CBC and TREX, we purified GST alone, GST-Aly, GST-UAP56, and GST-eIF4A3 (Figure 6A) and used equivalent amounts of these proteins for GST pull-downs from RNase-treated

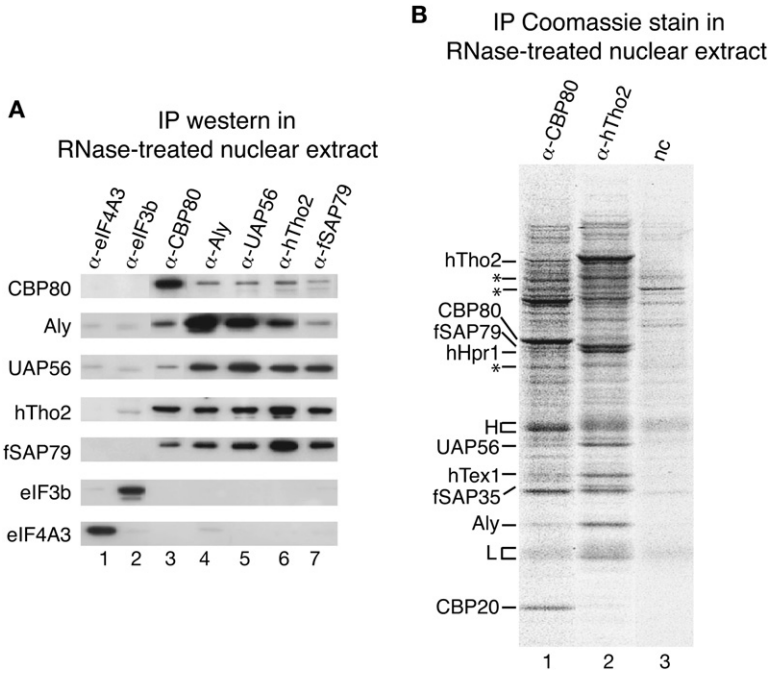

Figure 5. CBC Interacts with TREX

(A) Immunoprecipitations from RNase-treated nuclear extracts were performed using indicated antibodies followed by western analysis. (B) Immunoprecipitates from RNase-treated nuclear extracts using CBP80 antibody (lane 1), hTho2 antibody (lane 2), or negative control (nc) antibody were run on a 4%–12% gradient gel and stained with Coomassie brilliant blue. Proteins were identified by mass spectrometry. H indicates heavy chain and L indicates light chain. Asterisks indicate nonspecific bands.

nuclear extract. All of the TREX components were detected in GST-Aly and GST-UAP56 pull-downs as reported (Masuda et al., 2005, Figure 6B, lanes 2 and 3) but were not associated with GST-eIF4A3 or GST alone (lanes 1 and 4). Significantly, western analysis showed that CBP80 is present in the GST-Aly and GST-UAP56 pull-downs, but not in GST-eIF4A3 or GST alone pull-downs (Figure 6C, lanes 1–4). These data provide additional evidence that CBC is specifically associated with TREX but not with the EJC.

To determine whether Aly or UAP56 mediates the interaction between CBC and TREX, we in vitro translated CBP80, CBP20, and luciferase (as a negative control protein). GST pull-downs were carried out using equal amounts of GST-Aly, GST-UAP56, or GST-eIF4A3 (Figures 6A and 6D). Strikingly, CBP20 and CBP80 but not luciferase were pulled down by GST-Aly (Figure 6D, lane 2; see darker exposure in lower panel for CBP20). In contrast, none of the in vitro-translated proteins were pulled down by GST-UAP56 or GST-eIF4A3 (Figure 6D, lanes 3 and 4). To determine whether CBP80 and/or CBP20 is required for the interaction between CBC and Aly, GST pull-downs were carried out from a mixture of in vitro-translated CBP80 and luciferase or CBP20 and luciferase (Figure 6D, lanes 5–10). As shown in Figure 6D (lanes 5–7), CBP80 is pulled down by GST-Aly in the absence of CBP20 (lanes 5–7). Consistent with this observation, CBP20 alone is not pulled down by GST-Aly (lanes 8–10). Together, these data indicate that the association of CBC with TREX occurs via a protein-protein interaction between CBP80 and Aly. This result fits well with our observation that Aly is the TREX component bound closest to cap on the mRNA. As shown above (Figure 6C, lane 3), CBP80 is associated with GST-UAP56 in RNase-treated HeLa nuclear extract. Together, our data indicate that the Aly present in the extract mediates the interaction between UAP56 and CBP80.

A Role for CBP80 in mRNA Export

U snRNAs as well as mRNAs are capped at their 5′ ends and bind to CBC (Izaurralde et al., 1992, 1994). Cap/CBC is required for U snRNA export but Aly/TREX is not. Instead, U snRNA export occurs via the PHAX/Crm1/RanGTP pathway, and PHAX mediates the interaction with CBC (Fornerod et al., 1997; Ohno et al., 2000). In light of our observation that Aly and CBP80 interact, we next sought to investigate the role of CBP80 in mRNA export using functional assays. To do this, we microinjected excess U1 snRNA into *Xenopus* oocytes and assayed the effects on mRNA export (Figure 7A). tRNA, which does not use CBP80 for export, was used as a control. Significantly, these data revealed that excess U1 snRNA strongly inhibits mRNA export without affecting tRNA export (Figure 7A). These results support the conclusion that CBC/CBP80 is a common factor in U1 snRNA and mRNA export.

As another approach to address the role of CBP80 in mRNA export, we analyzed a mutant form of Aly that lacks 16 C-terminal amino acids (Figure 7B). This protein, AlyΔC, does not bind at all to UAP56, whereas wild-type Aly forms a tight complex with UAP56 (Luo et al., 2001). To determine whether AlyΔC binds to CBP80, we expressed GST-Aly, GST-AlyΔC, and negative control proteins (GST and GST-eIF4A3) in *E. coli* (Figure 7B, lanes 1–4). These proteins were used for pull-downs from RNase-treated nuclear extract. Western analysis revealed

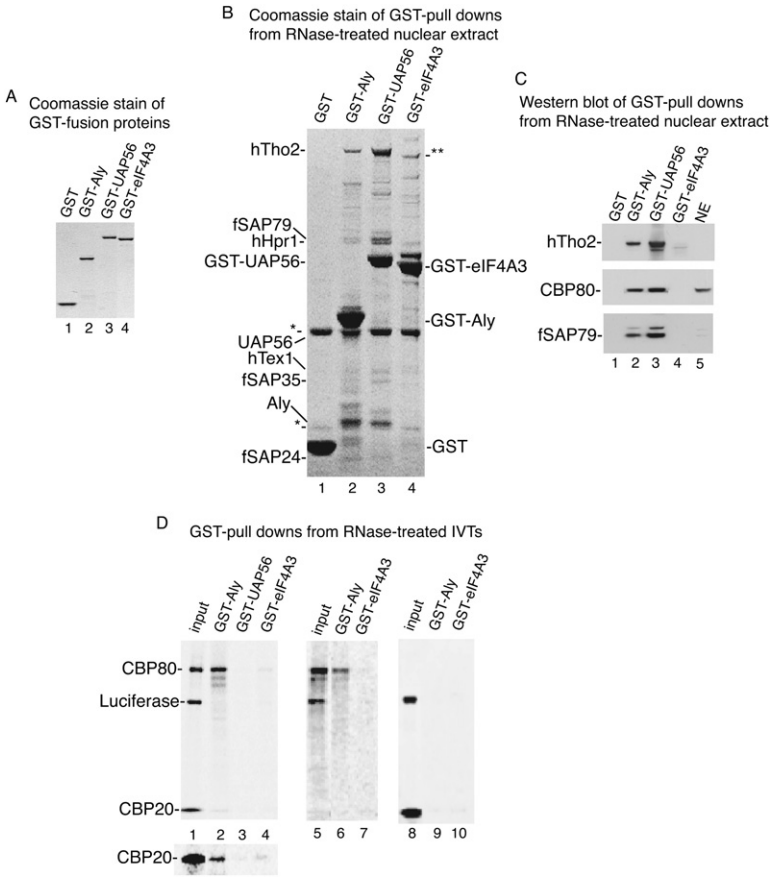

Figure 6. Aly Mediates the Interaction between CBP80 and TREX

(A) Purified GST-fusion proteins.

(B) GST pull-downs from RNase-treated nuclear extracts. Single asterisks indicate GST-binding proteins in nuclear extract. The double asterisk indicates eIF3a.

(C) Western analysis of GST pull-downs in (B). Nuclear extract (NE) (1 μl) was loaded.

(D) GST pull-downs from RNase-treated CBP80/CBP20 co-in vitro translation (IVT) mixed with luciferase IVT (lanes 1–4), CBP80 IVT mixed with luciferase IVT (lanes 5–7), or CBP20 IVT mixed with luciferase IVT (lanes 8–10). One-twentieth of input was loaded. The lower left panel is a darker exposure to show CBP20.

that CBP80 binds to AlyΔC as avidly as to wild-type Aly but does not bind to the negative control proteins (Figure 7C, lanes 1–4). Thus, we next used the *Xenopus* oocyte microinjection assay to ask whether excess Aly or AlyΔC affects export of mRNA, U1 snRNA, or tRNA (Figures 7D and 7E). Significantly, when Aly or AlyΔC was injected, U1 snRNA export was inhibited, whereas tRNA export was as efficient as when the negative control protein was injected (Figure 7D; see Figure 7E for a lighter exposure showing U1 snRNA export). As shown in Figure 7D, AlyΔC also inhibits mRNA export. In contrast, as expected from previous studies (Rodrigues et al., 2001; Zhou et al., 2000), wild-type Aly stimulates mRNA export (Figure 7D). Together, these data indicate that Aly and AlyΔC bind to CBP80, thereby functioning as dominant-negative inhibitors of U1 snRNA export. Only AlyΔC, which binds CBP80 but cannot bind UAP56, is a dominant-negative inhibitor of mRNA export. These data provide further evidence that CBP80 functions in mRNA export and thus is a common factor in the otherwise distinct mRNA and U1 snRNA export pathways.

DISCUSSION

In this study, we have obtained evidence for a new model for mRNA export. According to the current model, the mRNA export machinery is a structural and functional component of the EJC that binds to mRNA in a splicing-dependent manner at each exon-exon junction (Gatfield et al., 2001; Le Hir et al., 2000a, 2001). The EJC also contains factors required for NMD (Chan et al., 2004; Ferraiuolo et al., 2004; Gatfield et al., 2003; Gehring et al., 2003; Kim et al., 2001a, 2001b; Lykke-Andersen et al., 2001; Palacios et al., 2004; Shibuya et al., 2004; Singh and Lykke-Andersen, 2003). The current model also contends that the cap is not required for mRNA export (for review, see Moore, 2005).

We initiated our study by investigating the binding site on the mRNA of the THO complex, which has a conserved role in mRNA export (Gatfield et al., 2001; Herold et al., 2003; Luo et al., 2001; Rehwinkel et al., 2004; Strasser et al., 2002; Zhou et al., 2000; Jimeno et al., 2002; Le Hir et al., 2001; Rodrigues et al., 2001; Strasser and Hurt, 2001; Stutz et al., 2000) and interacts with Aly and UAP56 to form the TREX complex (Masuda et al., 2005). Unexpectedly, our analysis revealed that the entire TREX complex, including Aly and UAP56, does not bind at the EJC but instead binds upstream of it and is only bound to the first exon rather than every exon. RNAP II transcripts, including U snRNAs and mRNAs, contain a 5′ cap that is tightly bound by the heterodimeric CBC (CBP80 and CBP20). Our unexpected observation that

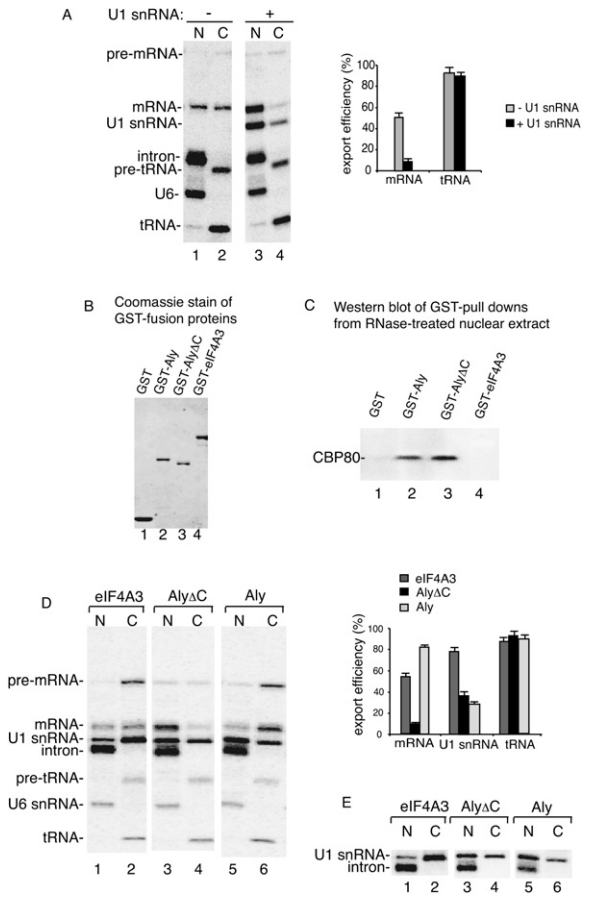

Figure 7. A Role for CBP80 in mRNA Export

(A) A mixture of AdML pre-mRNA, pre-tRNA, and U6 snRNA without (lanes 1 and 2) or with (lanes 2 and 3) excess U1 snRNA (~50-fold molar excess over pre-mRNA) was microinjected into oocyte nuclei followed by incubation for 3.5 hr. RNA from the nucleus (N) or cytoplasm (C) was analyzed. The export efficiency, defined as the amount of RNA in the cytoplasm divided by the total RNA at time 0 (data not shown), is shown in the graph. Quantitation of three independent experiments is shown. Error bars represent standard deviations (n = 3).

(B) Purified GST-fusion proteins.

(C) Western analysis of GST pull-downs from RNase-treated nuclear extract using the CBP80 antibody.

(D) One picomole each of GST-Aly, GST-AlyΔC, or GST-4A3 (the negative control) was microinjected into *Xenopus* oocyte cytoplasm followed by overnight incubation to import the GST proteins. A mixture of AdML pre-mRNA, U1 snRNA, pre-tRNA, and U6 snRNA was injected into the nuclei of these oocytes, followed by incubation for 3.5 hr. RNA from the nucleus (N) or cytoplasm (C) was analyzed. The export efficiency (%) is defined as in (A). Quantitation of three independent experiments is shown. Error bars represent standard deviations (n = 3).

(E) A lighter exposure of a portion of the gel shown in (D) to show the export of U1 snRNA.

TREX is located near the 5′ end of mRNA prompted us to ask whether the cap plays a role in recruitment of TREX. Our analysis revealed that TREX recruitment is indeed cap dependent, a finding that led us to revisit the question of whether the 5′ cap functions in mRNA export. In the original study of this question (Hamm and Mattaj, 1990), the cap was found to have a role in both U1 snRNA and mRNA export, but in subsequent work it was concluded that the cap only functions in U1 snRNA export (Izaurralde et al., 1995; Jarmolowski et al., 1994; Lewis and Izaurralde, 1997; Masuyama et al., 2004). Our data show that the cap does have a role in mRNA export but only for spliced mRNAs and not for cDNA transcripts or random RNAs. This observation reconciles the discrepancy concerning the previous studies of the cap, as spliced mRNA was used in the original study (Hamm and Mattaj, 1990), and in subsequent work cDNA transcripts were used (Izaurralde et al., 1995; Jarmolowski et al., 1994; Lewis and Izaurralde, 1997; Masuyama et al., 2004).

After establishing a role for the cap in both TREX recruitment and mRNA export, we asked whether CBC has a role in recruitment and/or export. Significantly, our data revealed that CBC is abundantly associated with TREX. Furthermore, we found that this association occurs via a protein-protein interaction between CBP80 and Aly, which is the TREX component bound on mRNA closest to the cap. Finally, in functional export assays, we found that excess U1 snRNA is a potent inhibitor of mRNA export and, conversely, Aly/AlyΔC is a dominant-negative inhibitor of U1 snRNA export. Thus, these data indicate that CBP80/CBC is a factor common to the U snRNA and mRNA export pathways.

5′ to 3′ Directionality for mRNA Export

The location of TREX at the 5′ end of mRNA provides a biochemical explanation for electron microscopic studies showing that the mRNPs of the giant Balbiani rings of *Chironomus tentans* translocate through the nuclear pore in a 5′ to 3′ direction (Daneholt, 1997; Mehlin et al., 1992; Visa et al., 1996a; see model, Figure 8A). CBC is detected on the 5′ end of the Balbiani ring mRNP and is coexported with it to the cytoplasm (Visa et al., 1996a, 1996b). The Balbiani ring data also indicate that the ribosome associates with the mRNA as it emerges from the nuclear pore (Daneholt, 1997; Mehlin et al., 1992; Visa et al., 1996a). NMD, in which the EJCs function to trigger mRNA degradation if a premature termination codon is detected, is thought to occur when the reading frame is first read (Chiu et al., 2004; Ishigaki et al., 2001; Lejeune et al., 2002). Thus, it is possible that as the mRNA exits the pore in a 5′ to 3′ direction and is mediated by TREX, each of the EJCs located at the exon-exon junctions is scanned in a 5′ to 3′ direction during the pioneer round of translation (Figure 8A). A topic of general debate has been whether NMD occurs in the nucleus or cytoplasm (for review, see Dahlberg and Lund, 2004). It is possible that this issue has been difficult to resolve, in part because NMD occurs as the mRNA is exiting the pore and thus is partially associated with both nuclear and cytoplasmic compartments. Our data also raise the possibility that binding of the ribosome as the mRNA emerges from the pore plays a role both in assisting exit of the mRNA from

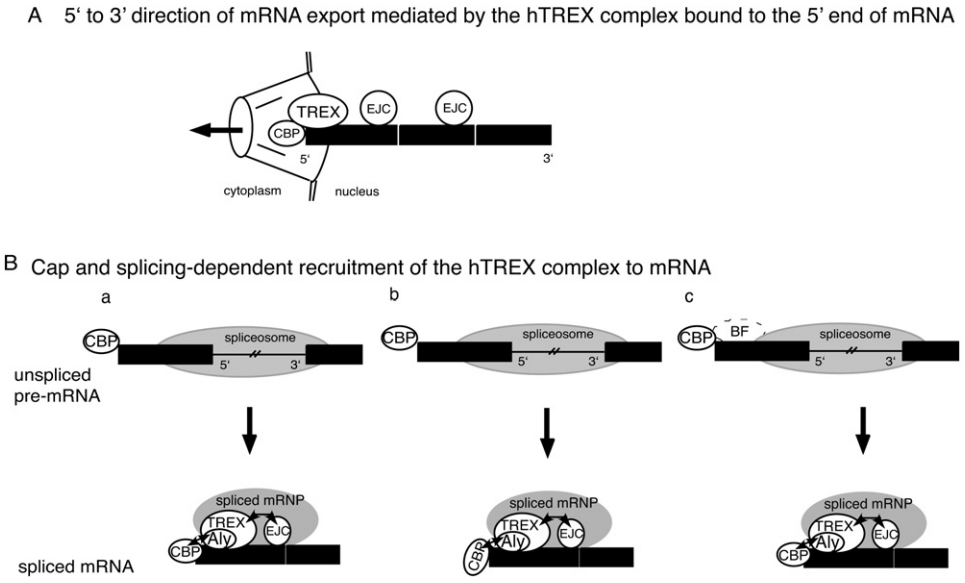

Figure 8. Model for mRNA Export

(A) Model for 5′ to 3′ translocation of mRNA. An mRNA containing three exons is shown with the cap-binding complex (CBC) bound to the 5′ end. An EJC is bound at each exon-exon junction, and TREX is bound at the 5′ end of the mRNA. The mRNA is exported in a 5′ to 3′ direction due to the presence of TREX at the 5′ end of the mRNA, and TREX may interact with the mRNA export receptor Tap/p15 located at the nuclear pore complex. (B) Models for cap- and splicing-dependent recruitment of the human TREX complex. See text for details.

the pore and/or in preventing the mRNA from returning to the nucleus.

Model for Cap- and Splicing-Dependent Recruitment of the mRNA Export Machinery

It is well-known that cap/CBC is present on unspliced pre-mRNA (Visa et al., 1996b), yet TREX is not recruited to unspliced pre-mRNA. The cap/CBC is also present on cDNA transcripts, yet TREX is not efficiently recruited to them either. Possible mechanisms to explain this cap- and splicing-dependent recruitment/export are shown in Figure 8B. In one model, splicing is important because a component of the spliced mRNP, such as the EJC, stabilizes TREX on spliced mRNA (Figure 8Ba). Thus, in the absence of splicing (i.e., on cDNA transcripts), TREX may be recruited via CBP80-Aly interactions but may not be stably bound to the mRNA due to lack of interaction with a splicing-specific factor bound only to spliced mRNPs. It is also possible that splicing causes some type of change, such as a conformational change that facilitates the CBP80-Aly/TREX interaction (Figure 8Bb). Finally, a blocking factor (BF), possibly the spliceosome itself or an associated factor, may prevent CBP80-dependent recruitment of TREX to unspliced pre-mRNA until a rearrangement occurs during splicing (Figure 8Bc). As cDNA transcripts are expected to lack this BF, other splicing-dependent mechanisms must exist to ensure stable recruitment of TREX to spliced mRNA (e.g., stabilization of TREX via interactions with spliced mRNP component[s]). The models proposed are not mutually exclusive, and these or related mechanisms may all play a role in the cap- and splicing-dependent recruitment of TREX to the 5′ end of mRNA. Previous work indicated that the metazoan TREX complex functions in export of not only spliced mRNAs but also mRNAs derived from genes that naturally lack introns (Gatfield et al., 2001). Thus, it will be of interest to determine whether the cap plays a role in export of naturally intronless mRNAs and whether TREX is recruited to the 5′ end.

EXPERIMENTAL PROCEDURES

Plasmids

Plasmids encoding AdML and Ftz pre-mRNA or the corresponding cDNAs were digested with BamHI or XhoI, respectively (Luo et al., 2001), and transcribed with T7 RNA polymerase. To generate antisense transcripts (used in Figure S2), these plasmids were cut with EcoRV and PstI, respectively, and transcribed with SP6 RNA polymerase. An EcoRI-XbaI fragment containing the first four exons of the *Xenopus laevis* Smad gene was inserted into the same sites of the pCS2 vector (Invitrogen). PCR was used to insert the AdML intron into Smad exon-exon junctions. To obtain the template for in vitro transcription with SP6 RNA polymerase, PCR was performed to amplify the fragment containing the SP6 promoter and the first three exons. The plasmid-containing histone H2a cDNA was cut with EcoRI and transcribed with T7 polymerase to generate the antisense transcript. Plasmids encoding GST-Aly, GST-AlyΔC, and GST-eIF4A3 were described (Bruhn et al., 1997; Luo et al., 2001; Ferraiuolo et al., 2004).

Antibodies

Rabbit antibodies to human CBP80, eIF4A3, and Y14 were raised against the peptides MSRRRHSDENDGGQPHKRR, ATSGSARKRL LKEED, and DESIHKLKEKAKKRKGRGFGSE, respectively (Genemed Synthesis, Inc.). Antibodies to Aly, UAP56, hTho2, and fSAP79 were described (Ferraiuolo et al., 2004; Luo et al., 2001; Masuda et al.,

2005; Zhou et al., 2000). The antibody to eIF3b was from Santa Cruz Biotechnology, Inc. The negative control antibody is against SAP 130.

RNA Immunoprecipitations

Pre-mRNAs were incubated for 2 hr in splicing reaction mixtures (25 μl) containing 30% HeLa nuclear extract (Krainer et al., 1984). Oligonucleotide-directed RNase H cleavage and immunoprecipitation (Le Hir et al., 2000a) was accomplished by adding each DNA oligonucleotide to a final concentration of 0.5 μM to in vitro splicing reactions and incubating for 10 min at 30°C. For immunoprecipitations, 5 μl of splicing reaction and 100 μl of binding buffer (20 mM Hepes [pH 7.9], 150 mM [or 250 mM] KCl, 0.1% Triton, 2.5 mM EDTA, and 5 mM DTT) were mixed with 10 μl protein A Sepharose beads, which were coupled with 5 μl of antibody. Immunoprecipitations were performed at 4°C for 2 hr in 150 mM KCl except in Figure 2, where 250 mM KCl was used. Beads were washed four times with 1 ml of binding buffer. For each wash, beads were left on ice for 5 min. Immunoprecipitates were treated with Proteinase K at 37°C for 10 min, and immunoprecipitated RNAs were recovered by phenol/chloroform extraction and ethanol precipitation. RNA was analyzed on denaturing polyacrylamide gels and visualized by PhosphorImager. One-fourth of the input was loaded.

Affinity Purification of mRNPs

Spliced mRNPs were assembled on AdML pre-mRNA or the corresponding cDNA transcript containing three MS2-binding sites. MS2/MBP affinity chromatography was carried out in low (60 mM) salt (Zhou et al., 2002). The same procedure was also carried out using uncapped AdML pre-mRNA containing three MS2-binding sites. For negative control purifications, the RNAs lacked MS2-binding sites.

Protein Immunoprecipitations

Antibodies were covalently crosslinked to protein A Sepharose beads in dimethylpimelimidate (Sigma) at a 2:1 ratio of beads to antibody (Harlow, 1988). Nuclear extract (200 μl) was incubated under splicing conditions for 30 min and then mixed with 5 volumes of buffer A (Harlow, 1988), protease inhibitor EDTA-free (Roche), and 20 μl of antibody-crosslinked beads. Samples were run on a 4%–12% gradient gel (Invitrogen), stained with Coomassie brilliant blue, and proteins were identified by mass spectrometry (Gygi et al., 1999).

GST Pull-Downs from Nuclear Extract

GST-binding proteins were precleared from 200 μl of RNase-treated nuclear extract by mixing the extract with 200 μl of splicing dilution buffer (20 mM Tris-HCl pH 7.9 and 100 mM KCl), 400 μl of PBS/0.1% Triton X-100/0.2 mM PMSF (buffer A), protease inhibitor EDTA-free (Roche), 8 μg of GST protein, and 20 μl of glutathione Sepharose 4B (Amersham). The reaction mixture was rotated at 4°C for 2 hr. After a brief spin, the supernatant was transferred to a new tube and mixed with 20 μl of glutathione Sepharose 4B at 4°C for 2 hr to remove excess GST. After spinning, the supernatant was mixed with 8 μg of GST alone, GST-Aly, GST-UAP56, or GST-eIF4A3 and 20 μl of glutathione Sepharose 4B at 4°C overnight. The beads were washed four times with buffer A and eluted with SDS loading buffer containing 0.5 mM DTT. After boiling, samples were loaded on a 4%–12% gradient gel (Invitrogen) followed by staining with Coomassie brilliant blue or western analysis.

GST Pull-Downs of In Vitro-Translated Proteins

[^{35}S] methionine-labeled CBP80, CBP20, and luciferase were produced using the TNT T7 quick-coupled transcription/translation kit (Promega).

CBP80 and CBP20 were co-in vitro translated or individually in vitro translated, mixed with in vitro-translated luciferase, and then incubated with RNase A to a final concentration of 0.35 ng/μl at 30°C for 20 min. Ten microliters of this reaction mixture was incubated with 8 μg of GST-Aly, GST-UAP56, or GST-eIF4A3 protein, 500 μl of PBS/0.1% Triton X-100/0.2 mM PMSF (buffer A), protease inhibitor EDTA-free (Roche), and 20 μl of glutathione Sepharose 4B (Amersham) for 5 hr at 4°C. The beads were washed five times with buffer A and then eluted with SDS loading buffer containing 0.5 mM DTT. After boiling, the samples were loaded on a 4%–12% gradient gel (Invitrogen). Proteins were visualized by autoradiography.

Xenopus laevis Oocyte Microinjection

^{32}P-labeled pre-mRNAs or cDNA transcripts containing or lacking the 5′ cap were mixed with U6 snRNA in a buffer containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.6 mM DTT, and 0.5 unit/ml RNAsin then microinjected into nuclei (Hamm and Mattaj, 1990). tRNA and U1Δsm snRNA were included in the injection mixture where indicated. Oocytes were incubated at 18°C for the times indicated. After dissection, the total RNA in the nucleus and cytoplasm was analyzed on denaturing 8% polyacrylamide gels and quantitated by PhosphorImager. Blue dextran and U6 snRNA were used as controls for the accuracy of microinjection and oocyte dissection. GST proteins were microinjected into the cytoplasm of the oocytes and incubated overnight to allow nuclear import.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/cgi/content/full/127/7/1389/DC1/.

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