Supplemental Figures to accompany the article:

Polyadenylation releases mRNA from RNA polymerase II in a process that is licensed by splicing

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Figure S1

Polyadenylation releases RNA whose only attachment to the polymerase is through the CPA.
(A) Pull-downs of polyadenylated wildtype and 3′-splice-site-mutant RNAs were compared in an experiment carried out as for lanes 3 and 4 of Figure 4 except that pull-down Method 2 (as for Fig. 3A) was used. The average and range for two independent experiments is shown.
(B) The pull-down efficiencies for the poly(A) site-cleaved RNAs of Figure 3B were re-calculated without normalization to the RNase H-cut RNA in order to allow comparison directly to part A of Figure S1.

Overview: This figure addresses the same question as Figure 4, but by a different approach. In Figure 4 the pull-down efficiencies of polyadenylated and non-polyadenylated 3′ splice site mutant RNAs were compared to each other in the same experiment, and the polyadenylated RNA was found to be pulled down much less efficiently, indicating that polyadenylation reduces the affinity of poly(A) site-cleaved RNA for the polymerase. However, the necessity of using oligo(dT) selection for the polyadenylated RNA (lanes 3 and 4), and the impossibility of using it for the non-polyadenylated RNA (lanes 1 and 2), required making direct comparisons between samples that had not been treated identically. We therefore sought to confirm this result using a different approach, in which the results for polyadenylated and non-polyadenylated 3′ splice site mutant RNAs were first normalized to their wildtype counterparts before being compared to each other. This approach is based on the finding in Figure 3A (and confirmed in Fig. S2) that 3′ splice site containing RNA binds equally to the polymerase whether polyadenylated or not. Therefore, polyadenylated wildtype RNA can be used for the normalization when oligo(dT) selection is required (Figure S1A), and non-polyadenylated wildtype RNA can be used for normalization when oligo(dT) selection is not possible (Figure S1B). This analysis confirmed the results of Figure 4 which showed that the pull-down efficiency of polyadenylated CPA-attached RNA is only one third that of non-polyadenylated CPA-attached RNA. Therefore, polyadenylation causes release of CPA-attached RNA from the polymerase.

Analysis: Figure S1A shows that polyadenylated RNA having a mutant 3′ splice site is pulled down substantially less efficiently by the polymerase than wildtype polyadenylated RNA (pull-down efficiency ratio = 0.27). This consequence of mutating the 3′ splice site is potentially the sum of two independent effects. The first of these is the effect of polyadenylation on the CPA to reduce the strength of RNA attachment to the polymerase. The second effect arises from the loss of direct 3′ splice site attachment to the polymerase because of the lack of 3′ splice site binding proteins in the CPA of the mutant RNAs. In order to focus on the first of these effects we need to dissect away the second.

As noted in the main text, in relation to Figure 3B, 3′ splice site binding proteins do mediate some attachment to the polymerase. To estimate the relative importance to polymerase binding of the 3′ splice site binding proteins within the EDC, we compared the pull-down efficiencies for wildtype and mutant poly(A) site-cleaved RNAs directly to each other (Figure S1B). This comparison was carried out using the same data set as for Figure 3B but without normalization to the RNase H-cut RNA as was done in that figure. This normalization was useful in Figure 3B to establish clearly the ability of the CPA alone to attach RNA to the polymerase and to show that

little else contributes to this attachment. But the normalization does not permit a direct comparison of the CPA to the EDC, as required here, because the two RNase H-cut RNAs differ from each other in having wildtype or mutant 3′ splice sites. Figure S1B shows, by means of a direct comparison of pull-down efficiencies, that the CPA pulls down poly(A) site-cleaved but non-polyadenylated RNA (lanes 3-4) about 77% as well as does the EDC (lanes 1-2). Thus, the absence of 3′ splice site binding proteins in the CPA reduces somewhat its affinity for the polymerase compared to the EDC (Figure S1A).

Since only 77% of the pull-down efficiency ratio for the EDC is attributable to the CPA we normalized the result from Figure S1A (0.27) to the result from Figure S1B (0.77). This allowed us to extract the poly(A)-dependent contribution to the decrease in pull-down efficiency of Figure S1A from the combined effects of polyadenylation and the loss of 3′ splice site binding proteins. The overall result is thus the pull-down efficiency ratio for polyadenylated vs. non-polyadenylated CPA-attached RNA—which is exactly the experiment of Figure 4. The numerical results of this analysis and that of Figure 4 (0.35 and 0.31) are also essentially the same.

The results of Figure S1A also provide some unrelated information. They show that mutation of the 3′ splice site results in altered poly(A) tail length control and the production of much hyperadenylated RNA (compare lanes 2 and 4). The heterogeneity and added length of much of the RNA in lane 4 of Figure S1, that is caused by mutation of the 3′ splice site, is entirely due to the production of excessively long poly(A) tails. Mutation of the 3′ splice site has no effect on RNA length if poly(A) tail growth is prevented by use of 3′ dATP (Figure S1B). A quantitative comparison in Figure S1 of the wildtype supernatant band (lane 2) with the mutant supernatant band (lane 4) shows that 50% of 3′ splice site mutant RNA is hyperadenylated.

The need for a functional 3′ splice site to maintain poly(A) tail length control was unexpected because CPSF, PAP and poly(A) binding protein II are the only factors reportedly required for this function. However, a role for the 3′ splice site in poly(A) tail length control is consistent with the fact that U2AF 65 and the U2 snRNP are known to interact functionally with PAP and CPSF, respectively, within the EDC (Vagner et al., 2000; Ko and Gunderson, 2002; Kyburz et al., 2006; Millevoi et al., 2006) (the U2AF-PAP interaction is not shown in Figure 1B). Apparently these interactions serve not only to abrogate polyadenylation-mediated release from the polymerase but also to stabilize the polyadenylation apparatus in a state that is competent to control poly(A) tail length.

Figure S2

The 3′-terminal EDC attaches intron-containing RNA to the polymerase and this RNA is not released by polyadenylation.

The experiment was carried out as for Figure 3A but using a splicing substrate that was splicing-inhibited by pre-incubation with 20 ng/µl of the DNA oligo CAGGTAAGTA (5′→3′) directed to the 5′ end of U1 snRNA (Black et al., 1985). Band 1 in lanes 3 and 4 was quantitated after subtracting the background appearing at the same position in lanes 1 and 2 respectively. The third exon oligo for RNase H cutting was CATTAGCCACACCAGCC (5′→3′) at 0.5 ng/µl which targeted the RNase H to a position 183 nt upstream of the poly(A) cleavage site. For the RNAP II pull-down the average and standard deviation for three independent experiments is shown. For the CstF pull-down the average and range for two independent experiments is shown. Lanes 1-4 and 5-6 are from different experiments. Note that although the overall pull-down efficiency is less in these experiments than in Figure 3A the numerical analysis, which includes a normalization to the RNase H-cut bands, still allows valid comparisons to be made.

We wanted to confirm for pβΔ-L that polyadenylation alone, as for pSV40E/L in Figure 3A, does not lead to release of EDC-attached RNA from the polymerase. We, therefore, compared the pull-down efficiencies of polyadenylated and non-polyadenylated pβΔ-L transcripts that had been

poly(A) site-cleaved but not spliced. The rationale for and analysis of the experiment with pβΔ-L were exactly as for Figure 3A and the results are shown in Figure S2 here. We decided to inhibit splicing in this experiment because the singly spliced processing intermediates of pβΔ-L (see Figures 5 and 6) migrate similarly to the RNase H-cut RNA to be used for normalization (Figure S2, band 3). Therefore, splicing was inhibited by including in the pre-incubation mixtures a DNA oligo that would direct RNase H to cut near the 5′ end of U1 snRNA (Black et al., 1985). Lanes 1-4 in Figure S2 confirm that polyadenylation of poly(A) site-cleaved, intron-containing RNA does not promote release from the polymerase (polyadenylated pull-down efficiency ÷ non-polyadenylated pull-down efficiency = 1.03 ± 0.07). We have also performed this experiment by mutating the 5′ splice site of the second intron instead of cutting the U1 snRNA with RNase H, again with similar results (data not shown). Mutating the 5′ splice site of the second intron prevents first or second intron singly spliced intermediates from being formed, as does cutting the U1 snRNA with RNase H, but by causing exon skipping (Rigo and Martinson, 2008) rather than by inhibiting splicing altogether.

We also confirmed that, as for the pSV40E/L transcripts of Figure 3A, CstF pulls down, and therefore remains associated with, a significant fraction of the poly(A) site-cleaved pβΔ-L transcripts (Figure S2, lanes 5 and 6, pull-down efficiency = 5.5). Interestingly, as is also true for the pSV40E/L transcripts of Figure 3A, CstF pulls down less of the RNase H-cut reference RNA than does the polymerase. This presumably reflects interactions with the polymerase, but not with CstF, of splicing proteins associated with the RNA.

Figure S3

The CPA attaches both mutant 3′ splice site RNA and cDNA transcripts to the polymerase.
(A) Mutant 3′ splice site transcripts. The experiment was like that of Figure 3B, lanes 3 and 4, except that the pull-down was by Method 2, to facilitate comparisons to part B (below). It confirms that the 3′ splice site associated proteins, as part of the EDC, do not contribute substantially to the attachment of poly(A) site-cleaved RNA to the polymerase. The average and standard deviation for three independent experiments is shown.
(B) cDNA transcripts. The cDNA construct was obtained by deleting the two introns of pβΔ-L successively using PCR. The deletions were confirmed by sequencing. The experiment was done as for lanes 1-2 of Figure S2 except that the α-U1snRNA oligo was not added. The average and range for two independent experiments is shown. The results in Figure S3B (band 3) show, in agreement with the results for the mutated pSV40E/L construct of Figure S3A (band 1), that the poly(A) site-cleaved RNA was efficiently pulled down by the polymerase even when it lacked any splice sites, while the RNase H-truncated RNA, which does not have a poly(A) site-cleaved 3′ end (band 4), was pulled down much less efficiently. Therefore, as for Figure S3A, this shows that proteins of the CPA that remain associated with the cleaved 3′ end of the RNA are sufficient to attach poly(A) site-cleaved RNA to the polymerase in the absence of any contributions from splice sites.

Although these results show that the CPA alone is *sufficient* to attach the RNA to the polymerase, a careful analysis of the data also indicates, as mentioned in connection with the CstF pull-downs (Figure S2), that splicing proteins associated with the pβΔ-L RNA do make some contribution to polymerase binding as well. Although splicing was inactivated in these experiments by cutting of the U1 snRNP RNA, this is not expected to prevent splicing complex formation at the 5′ splice site (Du and Rosbash, 2001). Thus, removing all introns and splice sites from the pβΔ-L transcripts caused the pull-down efficiency relative to their respective RNase H-cut transcripts to increase 2-fold (from 1.6 in lanes 1 and 2 of Figure S2 to 3.4 in Figure S3B). This reflects, most likely, less efficient pull-down by the polymerase of the RNase H-cut species after removal of the splice sites. The existence of splice site interactions with the polymerase, presumably through

spliceosomal complex formation on the pβΔ-L RNA, receives further support from the following comparison. Whereas the average pull-down efficiency relative to the RNaseH-cut normalization standard was 5.0 for pSV40E/L transcripts, whether polyadenylated or not, in Figure 3A, it was only 1.6 for pβΔ-L transcripts in Figure S2. This implies that the pβΔ-L RNase H-cut species (which has introns) binds more tightly to the polymerase than does the pSV40E/L RNase H-cut species. Although other interpretations of the data in Figures S2 and S3B are possible, the data of Figure 5 in the main text also suggest that spliceosomal complexes on pβΔ-L RNA make a minor contribution to polymerase binding. This conclusion comes from the analysis of splicing for each intron separately (as for Fig. 7) in the experiments leading to Figure 5. The results reveal a transcript pull-down efficiency mediated by the polymerase antibody of slightly less than 1 after first intron splicing (0.67 ± 0.01, average and range), consistent with the knowledge that spliceosomes associate with the polymerase CTD and that this association is disrupted by splicing (Zeng and Berget, 2000). This does not conflict with the overall pull-down efficiency for splicing both introns (0.95) that is given in Figure 5, because second intron splicing led to an increase in polymerase-mediated pull-down efficiency (but much less dramatic than for CstF in Fig. 7, see Fig. S4). This, when combined with the intron 1 decrease, gave an overall splicing-mediated change that was close to zero for both introns taken together (Fig. 5).

The data in Figure S3B also suggest that the exons of pβΔ-L promote some polymerase binding. This derives from the simple, though un-normalized, observation that the RNase H-cut pβcDNA-L transcripts in the pellet of Figure S3B (band 4, lane 1) are readily visible in the gel, unlike the RNase H-cut pSV40E/L transcripts in Figure S3A (band 2, lane 1). Moreover, the pellet/supernatant ratio is 6-fold less in Figure S3B than in Figure S3A (3.4 vs. 22). This shows that there is more binding by the RNase H-cut portion of the pβcDNA-L transcripts (which contain globin exons) than by the RNase H-cut portion of the pSV40E/L transcripts. Exon binding by the β-globin RNA probably derives from SR proteins bound to the first and second exons of these transcripts (Misteli and Spector, 1999; Schaal and Maniatis, 1999; Li and Manley, 2005; Das et al., 2007).

Figure S4

Antibodies directed to the polymerase CTD or to CstF 50 do not preferentially pull down second intron spliced RNA.

Pull-downs were performed using the monoclonal antibody 8WG16 (Covance) which recognizes the RNA polymerase II large subunit CTD, and the polyclonal antibody D-15 (Santa Cruz Biotechnology) raised against a peptide near the C-terminus of CstF 50. The control antibody was goat normal IgG. Lanes 1a, 3a, 5a and 7a are enhanced versions of lanes 1, 3, 5 and 7, respectively. Lanes 1-4 and 5-10 are from two different experiments. The pull-downs were by Method 2 for lanes 1 and 2, and by Method 1 for lanes 7-10. The 8WG16 is supplied as ascites fluid so we do not know how many μg of antibody are actually present for the pull-downs in lanes 3-6. The average and range are shown for the efficiencies with which the two first intron-spliced species (bands 2 and 4) and the two second intron spliced species (bands 3 and 4) were pulled down compared to their respective precursors (see Figure 7).

The CstF 64 pull-down in lanes 1 and 2 confirms the more than 3-fold preference (spliced/unspliced pull-down efficiency $= 3.4 \pm 0.8$) to co-IP second intron spliced (bands 3-4) vs. unspliced (bands 1-2) RNA presented in Figure 7. The formal, but extremely unlikely, possibility exists that the more efficient pull-down of second intron spliced RNA shown here and in Figure 7 reflects recruitment of additional CstF to spliced RNA rather than an increase in CstF 64 epitope availability. If, according to this possibility, the pull-down efficiency reflects only the number of CstF complexes present, then antibodies to a different subunit of CstF may be expected to similarly exhibit an increase in pull-down efficiency upon splicing. However, Figure S4 shows that a CstF 50

antibody does not show a preference for pulling down second intron spliced vs. unspliced RNA (spliced/unspliced pull-down efficiency = 1.09 ± 0.04). The simplest interpretation of these results is that a major conformational change occurs upon splicing that exposes CstF 64 epitopes but not the epitope recognized by the CstF 50 antibody.

A complete analysis of the data leading to Figure 5 (as for Fig. 7 but not shown in Fig. 5) indicates that with an antibody that recognizes the body of the polymerase there is a slight increase in pull-down efficiency upon second intron splicing (spliced/unspliced pull-down efficiency = 1.42 ± 0.02, average and range). We believe that, as for CstF 64, this reflects an increase in exposure of epitopes on the body of the polymerase. We anticipated that using an antibody whose epitopes are reiterated over a larger surface may reduce any effects of epitope availability. Lanes 3-6 of Figure S4 show that a pull-down with 8WG16 (which presumably can recognize multiple positions in the CTD of the polymerase) does not result in any preferential pull-down of second intron spliced RNA (spliced/unspliced pull-down efficiency for intron 2 is essentially 1.0 when averaged over two experiments). We conclude that, along with the increased exposure of epitopes on CstF 64, some epitopes on the body of the polymerase also become more exposed after splicing of the second intron.

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Figure S1

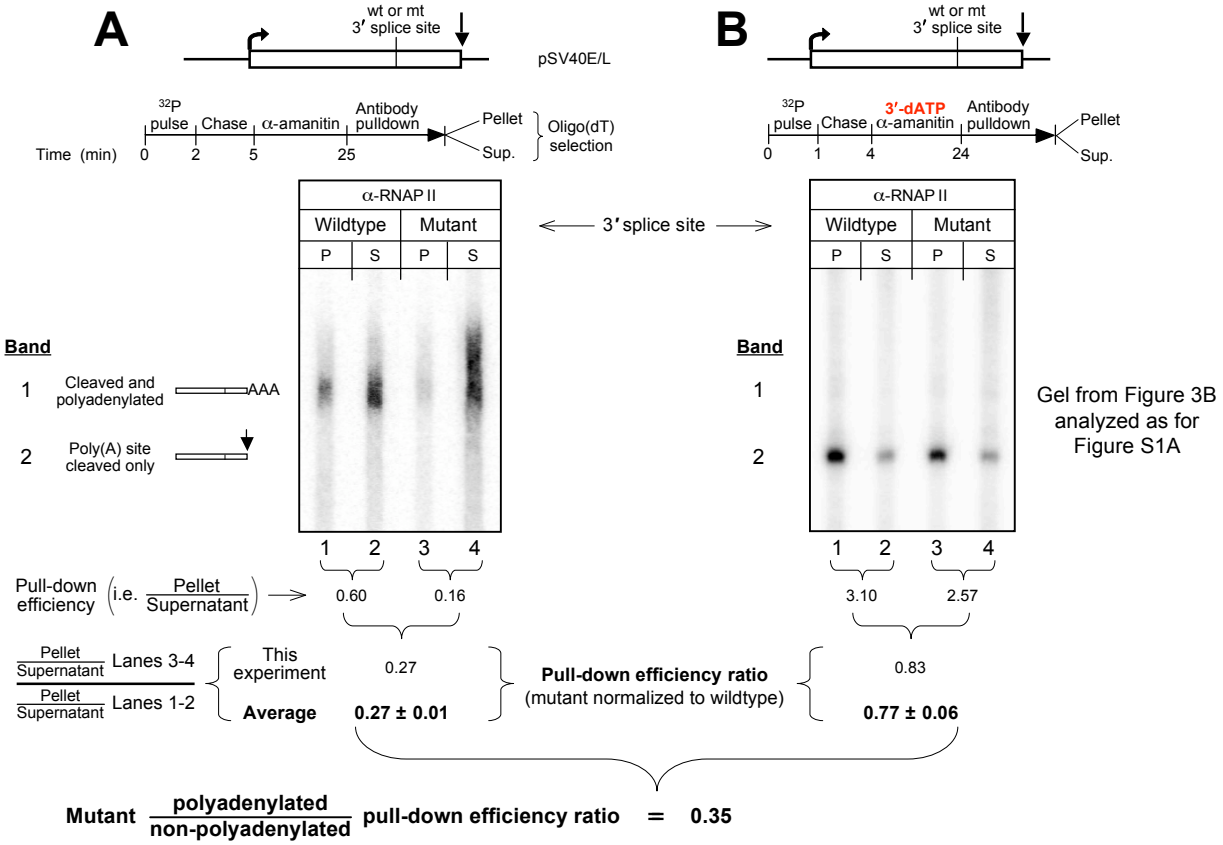

Figure S2

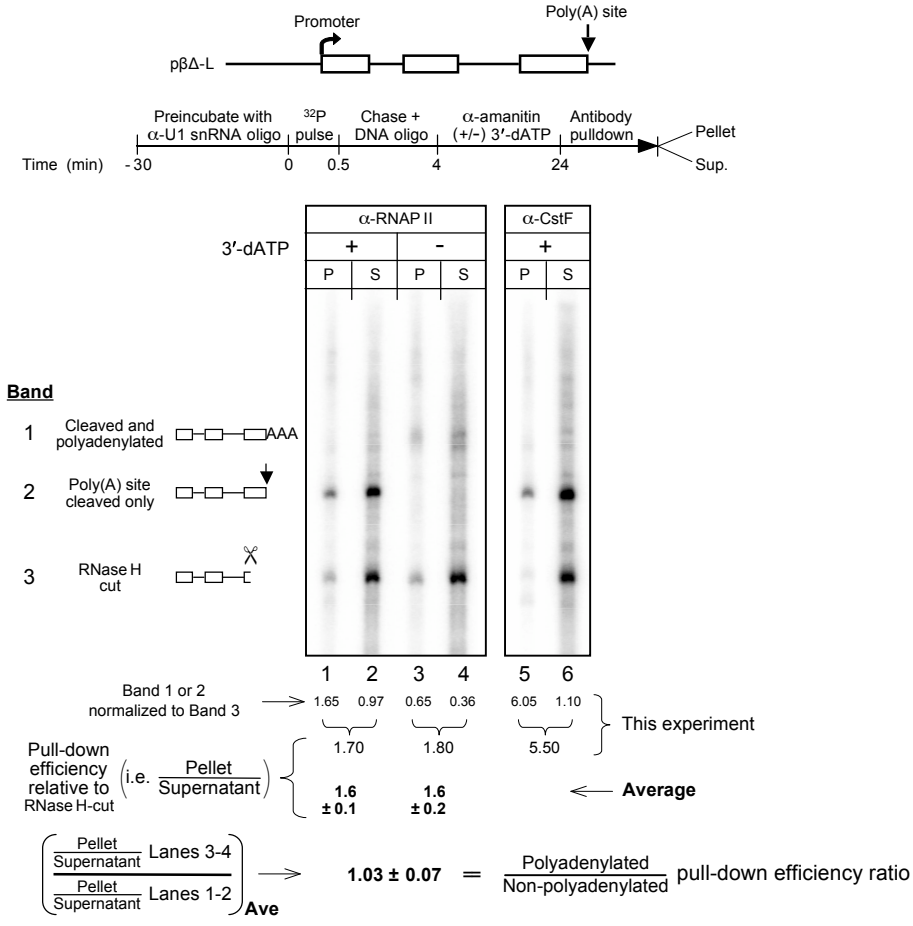

Figure S3

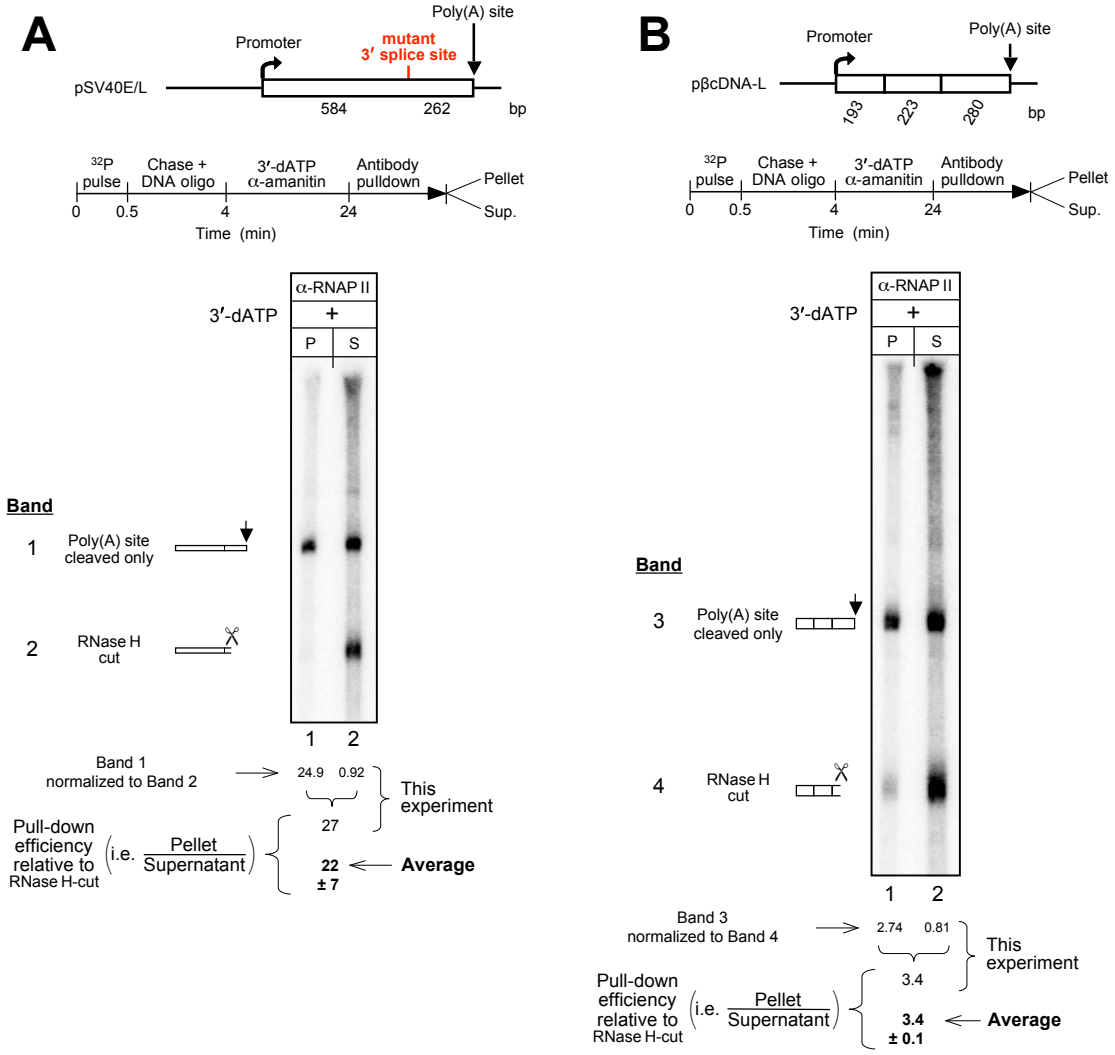

Figure S4