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Position-dependent inhibition of the cleavage step of pre-mRNA 3′-end processing by U1 snRNP

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ABSTRACT

The 3′ ends of most eukaryotic pre-mRNAs are generated by 3′ endonucleolytic cleavage and subsequent polyadenylation. 3′-end formation can be influenced positively or negatively by various factors. In particular, U1 snRNP acts as an inhibitor when bound to a 5′ splice site located either upstream of the 3′-end formation signals of bovine papilloma virus (BPV) late transcripts or downstream of the 3′-end processing signals in the 5′ LTR of the HIV-1 provirus. Previous work showed that in BPV it is not the first step, 3′ cleavage, that is affected by U1 snRNP, but rather the second step, polyadenylation, that is inhibited. Since in HIV-1 the biological requirement is to produce transcripts that read through the 5′ LTR cleavage site rather than being cleaved there, this mechanism seemed unlikely to apply. The obvious difference between the two examples was the relative orientation of the 3′-end formation signals and the U1 snRNP-binding site. In vitro assays were therefore used to assess the effect of U1 snRNP bound at various locations relative to a cleavage/polyadenylation site on the 3′ cleavage reaction. U1 snRNP was found to inhibit cleavage when bound to a 5′ splice site downstream of the cleavage/polyadenylation site, as in the HIV-1 LTR. U1 snRNP binding at this location was shown not to affect the recruitment of multiple cleavage/polyadenylation factors to the cleavage substrate, indicating that inhibition is unlikely to be due to steric hindrance. Interactions between U1A, U1 70K, and poly(A) polymerase, which mediate the effect of U1 snRNP on polyadenylation of other pre-mRNAs, were shown not to be required for cleavage inhibition. Therefore, U1 snRNP bound to a 5′ splice site can inhibit cleavage and polyadenylation in two mechanistically different ways depending on whether the 5′ splice site is located upstream or downstream of the cleavage site.

Keywords: 5′ splice site; BPV; cleavage/polyadenylation; HIV-1; pre-mRNA splicing

INTRODUCTION

Newly synthesized eukaryotic mRNA precursors have to be released from their site of transcription, processed by removal of intervening sequences, and protected from exonucleolytic degradation before they can be transported to the cytoplasm and translated there. All these posttranscriptional steps are influenced by the proper acquisition of the mRNA 3′ end. Most pre-mRNAs acquire a poly(A) tail at their 3′ end by cleavage and polyadenylation. This process occurs in two steps, an endonucleolytic cleavage of the RNA followed by the addition of the poly(A) tail. The RNA sequences that determine the precise position of cleavage in metazoa are a highly conserved AAUAAA hexanucleotide found upstream of the cleavage site and a more variable GU-rich sequence found downstream.

Although simple in principle, 3′-end processing of pre-mRNAs requires a surprisingly large number of proteins (Colgan & Manley, 1997; Wahle & Rüegsegger, 1999). The cleavage and polyadenylation specificity factor (CPSF) is a tetrameric complex whose 160-kDa subunit binds directly to the AAUAAA sequence. The 160-kDa subunit of CPSF interacts with the 77-kDa subunit of CstF (cleavage stimulation factor), a trimeric factor that binds to the GU-rich sequence via its 64-kDa subunit. CPSF and CstF bind to pre-mRNAs coopera-

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tively. Poly(A) polymerase (PAP) is the enzyme that generates the poly(A) tail and is recruited to the RNA via its interaction with CPSF-160. PAP is also required for the cleavage step on certain pre-mRNA substrates. Finally, two cleavage factors, CF I_m and CF II_m, are essential for cleavage. Whereas CF II_m is poorly characterized, CF I_m has been purified to homogeneity, and cDNAs encoding for two of its subunits have been isolated. CF I_m appears to act at a very early step in the reaction and may help in the recruitment of additional cleavage factors. After cleavage has occurred, the first phase of addition of the poly(A) tail requires only CPSF and PAP. Once 10–15 A residues have been added, poly(A) binding protein II binds to the short A stretch and supports processive synthesis of the remaining residues of the poly(A) tail (Colgan & Manley, 1997; Wahle & Rüegsegger, 1999).

Recent evidence indicates that the cleavage/polyadenylation factors do more than participate in 3′-end processing. Mammalian CPSF and CstF appear to associate with the carboxy-terminal domain of the RNA polymerase II large subunit to couple transcription and RNA processing (Dantonel et al., 1997; McCracken et al., 1997a, 1997b) and yeast cleavage factors were shown to direct transcription termination (Birse et al., 1998). Cleavage/polyadenylation sites have also been reported to stimulate splicing (Niwa & Berget, 1991; Gunderson et al., 1997) and it has been hypothesized that this stimulation could involve interaction between PAP and a still unknown splicing factor (Gunderson et al., 1997), an interaction that may be favored by weak binding of U1 snRNP to the substrate in the vicinity of the future 3′ end (Wassarman & Steitz, 1993). Reciprocally, a number of in vitro and in vivo studies have shown that splicing influences 3′-end processing (Niwa et al., 1990; Nesic et al., 1993; Wassarman & Steitz, 1993; Nesic & Maquat, 1994; Cooke & Allwine, 1996; Liu & Mertz, 1996; Antoniou et al., 1998; Baurén et al., 1998; Dye & Proudfoot, 1999), and that splicing factors interact with 3′-end processing factors. For example, the U1A protein of U1 snRNP stimulates polyadenylation of the SV40 late pre-mRNA via a direct interaction with the 160-kDa subunit of CPSF (Lutz et al., 1996).

There are also several well-understood examples of inhibitory effects of splicing factors on 3′-end formation. The U1A protein bound to its own pre-mRNA interacts with PAP to inhibit polyadenylation (Boelens et al., 1993; Gunderson et al., 1994). In the HIV-1 pre-mRNA, binding of U1 snRNP to the major splice donor site inhibits 3′-end formation at the cleavage and polyadenylation site located upstream of the donor site in the 5′ LTR of the HIV-1 provirus (Ashe et al., 1995, 1997). In the bovine papilloma virus (BPV) late pre-mRNA, U1 snRNP binding to a 5′ splice site located upstream of the major site of 3′-end formation late in infection inhibits use of this site early after infection (Furth & Baker, 1991; Furth et al., 1994). The mechanism in this case involves inhibition of the polyadenylation activity of PAP via direct interaction with the U1 70K protein of U1 snRNP (Gunderson et al., 1998). The HIV-1 and BPV examples are striking because they both involve the inhibition of 3′-end processing by U1 snRNP binding in the vicinity of a cleavage/polyadenylation site. However, the way U1 snRNP inhibits cleavage/polyadenylation in these two viruses is predicted to be different on the basis of the biological function of the regulatory process in each case. In BPV, the goal is to prevent accumulation of late mRNAs during early infection in spite of the fact that their precursors are being constantly transcribed. Inhibition of polyadenylation ensures that 3′-end cleaved, unstable RNAs are produced from the BPV late genes that are destined to be rapidly degraded. If cleavage was inhibited instead, the transcriptional readthrough products might be stabilized by the use of downstream 3′-end formation sites. The function of the inhibition of pre-mRNA cleavage in the HIV-1 5′ LTR is to allow transcriptional readthrough of the provirus. To produce viral mRNAs, as well as genomic RNAs, 3′-end formation must occur in the 3′ LTR and not in the 5′ LTR. If inhibition of 3′-end formation in the 5′ LTR were at the polyadenylation step, cleavage would take place and full-length viral pre-mRNA would still not be produced. This suggested that in HIV-1, the cleavage step should be targeted for inhibition, and not the polyadenylation step.

One obvious difference between the two systems is the position of U1 snRNP binding relative to the prospective site of 3′-end formation. We therefore examined whether the position of U1 snRNP binding relative to this site influenced the mechanism of inhibition of 3′-end formation. U1 snRNP binding to a 5′ splice site located downstream, rather than upstream, of a cleavage/polyadenylation site, is shown to affect cleavage rather than polyadenylation in vitro. Cleavage inhibition is not mediated by the PAP-U1 70K interaction that is required for inhibition of polyadenylation. Therefore, the relative positions on the pre-mRNA of U1 snRNP and the 3′-end processing factors appears to determine which interactions between the two complexes occurs, which in turn determines the mechanism of inhibition of 3′-end formation.

RESULTS

A 5′ splice site inhibits endonucleolytic cleavage at an upstream cleavage/polyadenylation site in HeLa nuclear extracts

To analyze the mechanism of HIV-1 poly(A) site inhibition by U1 snRNP observed in vivo (Ashe et al., 1995, 1997), we tried to establish in vitro cleavage assays in HeLa nuclear extracts that would recapitulate the regulation. However, previous studies have shown that the

HIV-1 poly(A) site is almost completely inactive in cell-free systems in the absence of activating sequences (Gilmartin et al., 1992; Valsamakis et al., 1992). As these sequences are not present in the context of the 5′ LTR poly(A) site, and because it was not expected from the work of Ashe et al. (1995) that the effects of U1 snRNP would be pre-mRNA-specific, we decided to utilize instead the well-characterized Adenovirus L3 cleavage and polyadenylation site. Pre-mRNA substrates were constructed in which a wild-type or a mutated 5′ splice site was inserted either 13, 52, or 68 nt downstream of an Adenovirus L3 cleavage/polyadenylation site (Fig. 1A). In all cases the wild-type 5′ splice site was AAG/GUAAGU and the mutant sequence AAG/caAAcU. In BPV, U1 snRNP binding upstream of the cleavage/polyadenylation site inhibits the polyadenylation step of 3′-end processing (Gunderson et al., 1998). To test whether U1 snRNP, when bound upstream of the cleavage/polyadenylation site, could also affect cleavage, pre-mRNA substrates were also utilized with a wild-type or mutant 5′ splice site positioned 7 nt upstream of the Adenovirus L3 cleavage/polyadenylation site (Gunderson et al., 1998; Fig. 1A).

Cleavage reactions in HeLa cell nuclear extracts were performed and showed that maximally 10–15% of the substrate RNAs were cleaved. Cleavage of the parent construct, AdL3, is shown in lanes 13 and 14 of Figure 1B. The cleavage efficiencies of the substrates containing a wild-type 5′ splice site downstream of the cleavage site were consistently three to six times lower than the cleavage efficiencies of the corresponding substrates with the mutant 5′ splice site (Fig. 1B; lanes 1–9). Inhibition was seen in all three constructs where the 5′ splice site was inserted in a downstream position relative to the cleavage site. This assay therefore recapitulates in vitro the inhibitory effect of the 5′ splice site

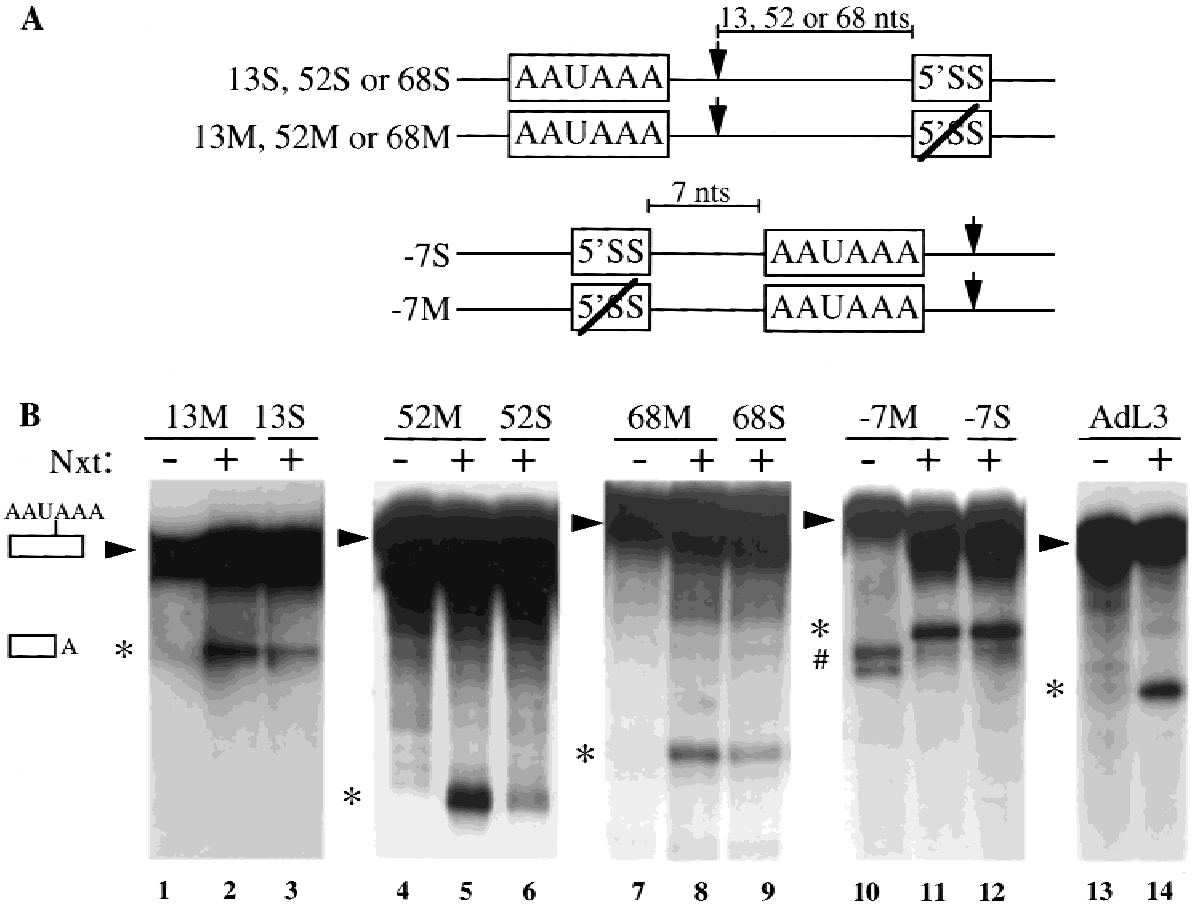

FIGURE 1. A 5′ splice site located downstream of a cleavage/polyadenylation site inhibits the cleavage step of 3′-end processing in HeLa cell nuclear extract. **A**: Scheme of the pre-mRNA substrates used. They contain the wild-type Adenovirus L3 cleavage/polyadenylation site (AAUAAA) either alone (AdL3) or with a wild-type (AAGGUAAGU) or a mutant (AAGcaAAcU) 5′ splice site (5′SS or slashed 5′SS, respectively) at various distances around the cleavage/polyadenylation site (the cleavage site is represented by an arrow). **B**: Effect of the presence of the 5′ splice site on cleavage of the pre-mRNAs in HeLa cell nuclear extract. Lanes 1, 4, 7, 10, and 13 were the input substrates incubated in complete reaction buffer without HeLa nuclear extract (Nxt), whereas lanes 2, 3, 5, 6, 8, 9, 11, 12, and 14 contained nuclear extract. After completion of the reaction, the RNAs were analyzed on a 10% denaturing polyacrylamide gel. The precursors are indicated with arrows, the 5′ cleavage product with asterisks and unspecific degradation products in lane 10 with #.

predicted from the in vivo observations on the HIV-1 5′ LTR poly(A) site. No effect of a 5′ splice site inserted upstream of the cleavage site in the -7S substrate was observed (Fig.1B; lanes 10–12), consistent with the previous conclusion that U1 snRNP mediates inhibition of polyadenylation, but not cleavage, in both this substrate (see below) and in the BPV pre-mRNA (Gunderson et al., 1998).

U1 snRNP inhibits 3′-end cleavage

To demonstrate that U1 snRNP binding to the 5′ splice site was responsible for the inhibitory effect on cleavage, the extract was pretreated with a U1-specific DNA oligonucleotide complementary to the 5′ end of U1 snRNA prior to the cleavage reaction. By virtue of the RNAse H present in the extract, the RNA strand of the RNA:DNA hybrid formed between the 5′ end of U1 snRNA and the U1 oligonucleotide was efficiently degraded, as determined by Northern blot (data not shown). The truncated U1 snRNA is unable to interact with the 5′ splice site (Krämer & Keller, 1990). For reasons that are not understood, addition of the U1 oligonucleotide increased the recovery of RNA from the extracts. However, when the proportion of cleaved RNA was measured, the efficiency of cleavage of the 13S substrate, with the wild-type 5′ splice site, was increased by fivefold (Fig. 2A,B; compare lanes 8 and 9 with 7). Similar results were observed with the substrates in which the 5′ splice site was located 52 or 68 nt downstream of the cleavage site (data not shown). No comparable effect was observed on cleavage of the control substrate with the mutant 5′ splice site (Fig. 2A,B; lanes 2–4) or with the -7S substrate (Fig. 2D, lanes 4 and 5). A further control, pretreatment of the extract with a DNA oligonucleotide directed against U2 snRNP, also had little effect on the cleavage efficiencies of the substrates with either the wild-type or mutated 5′ splice site (Fig. 2A,B: lanes 5 and 10; Fig. 2D, lane 3). To demonstrate that the lack of effect of U1 snRNP in the case of the -7S substrate is not due to its inability to bind to the 5′ splice site on this RNA, we verified that depletion of U1 snRNP but not U2 snRNP stimulates the polyadenylation activity on the -7S pre-mRNA, consistent with previous observations (Fig. 2C, lanes 2, 4, 5; Gunderson et al., 1998). We conclude that U1 snRNP

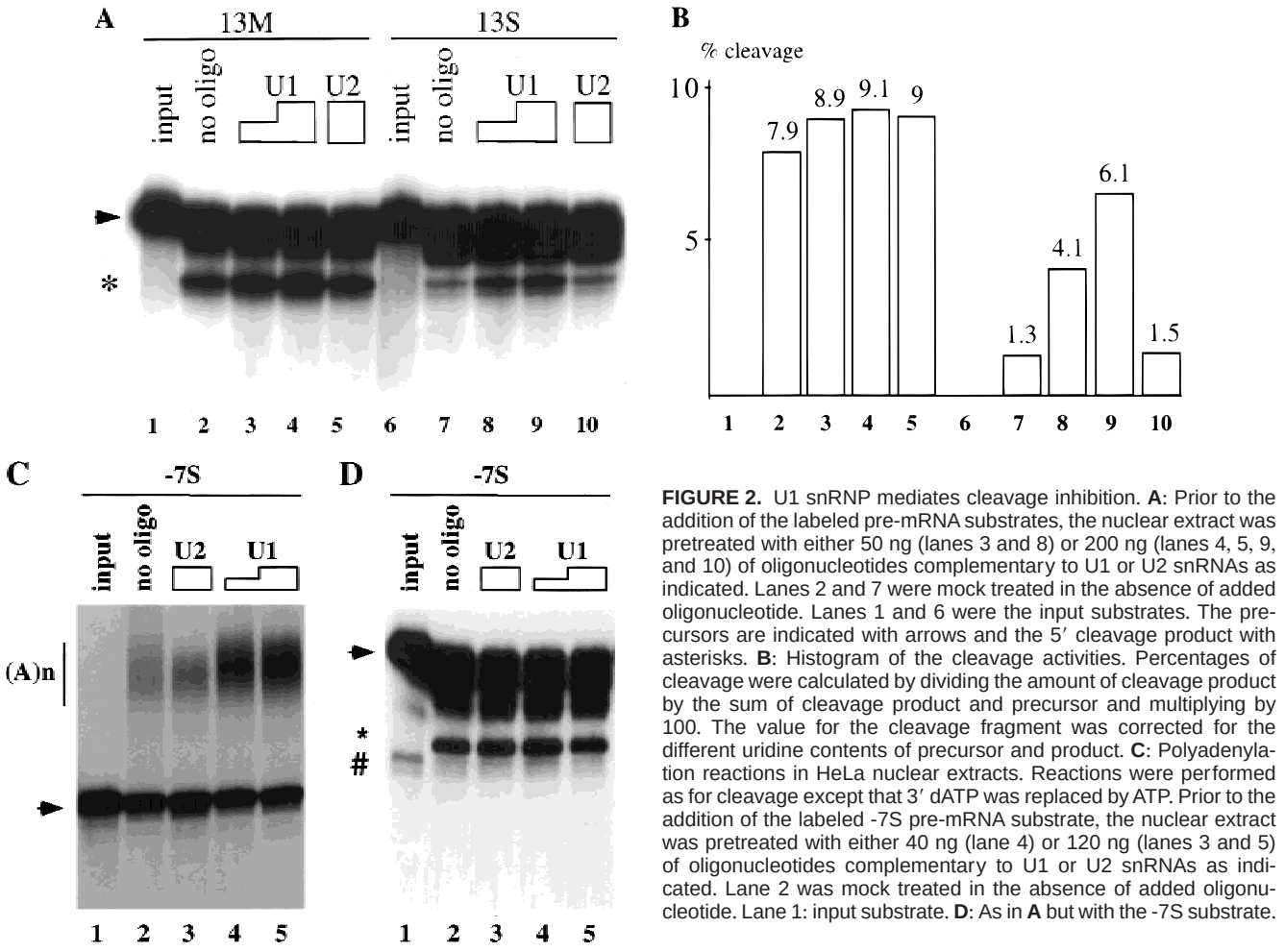

FIGURE 2. U1 snRNP mediates cleavage inhibition. **A**: Prior to the addition of the labeled pre-mRNA substrates, the nuclear extract was pretreated with either 50 ng (lanes 3 and 8) or 200 ng (lanes 4, 5, 9, and 10) of oligonucleotides complementary to U1 or U2 snRNAs as indicated. Lanes 2 and 7 were mock treated in the absence of added oligonucleotide. Lanes 1 and 6 were the input substrates. The precursors are indicated with arrows and the 5′ cleavage product with asterisks. **B**: Histogram of the cleavage activities. Percentages of cleavage were calculated by dividing the amount of cleavage product by the sum of cleavage product and precursor and multiplying by 100. The value for the cleavage fragment was corrected for the different uridine contents of precursor and product. **C**: Polyadenylation reactions in HeLa nuclear extracts. Reactions were performed as for cleavage except that 3′ dATP was replaced by ATP. Prior to the addition of the labeled -7S pre-mRNA substrate, the nuclear extract was pretreated with either 40 ng (lane 4) or 120 ng (lanes 3 and 5) of oligonucleotides complementary to U1 or U2 snRNAs as indicated. Lane 2 was mock treated in the absence of added oligonucleotide. Lane 1: input substrate. **D**: As in **A** but with the -7S substrate.

binding to a 5′ splice site downstream of the cleavage/polyadenylation site inhibits the cleavage step, whereas its binding upstream inhibits polyadenylation.

U1 snRNP inhibition in a reconstituted cleavage assay

A reconstituted cleavage assay can be performed by recombining purified fractions containing the various cleavage factors (Rüegsegger et al., 1996; see Materials and Methods). These fractions do not contain U1 snRNP (data not shown), providing a direct way to test the inhibitory effect of highly purified U1 snRNP on cleavage. Incubation of the various pre-mRNAs in this assay led to cleavage efficiencies of roughly 10% (Fig. 3; lanes 2, 7, 12). Addition of purified U1 snRNP to the reaction dramatically decreased the cleavage efficiency of the 13S RNA substrate (Fig. 3; lanes 3–5). This inhibitory effect was specific, as cleavage of the mutant control 13M substrate was not affected (Fig. 3; lanes 8–10), nor was cleavage of the -7S RNA substrate, which has a wild-type 5′ splice site located 18 nt upstream of the cleavage site (Fig. 3; lanes 12–14). As highly purified cleavage components and U1 snRNP were used, the inhibitory effect of U1 snRNP on 13S cleavage appears to be due to a direct effect of the snRNP on the 3′ cleavage factors.

U1 snRNP does not impair the binding of characterized cleavage factors

The simplest hypothesis to explain the observed inhibition was that U1 snRNP binding to the RNA downstream of the 3′ cleavage site would somehow impair the correct assembly of cleavage factors on the pre-mRNA. To test this, we first analyzed the binding of the 64-kDa subunit of CstF. Because CstF and CPSF bind cooperatively to pre-mRNA and form a stable ternary complex (see Introduction), the AAUAAA-dependent binding of the 64-kDa subunit of CstF to the pre-mRNA reflects the presence of these two factors. UV crosslinking experiments were performed with RNAs containing either a wild-type or a mutant Adenovirus L3 cleavage/polyadenylation site without a 5′ splice site (Fig. 4A; AAUAAA and AAGAAA, respectively) as well as with substrates derived from the AAUAAA construct with either a mutant or a wild-type 5′ splice site, the 13M and 13S RNAs respectively. The complex crosslinking patterns in HeLa nuclear extracts (Fig. 4A; lanes 1, 4, 7, and 10) were resolved by immunoprecipitation with a monoclonal antibody directed against CstF 64 (MacDonald et al., 1994) (Fig. 4; lanes 3, 6, 9, and 12). CstF 64 was bound to the RNA containing the wild-type but not the mutant cleavage/polyadenylation site (Fig. 4; lanes 3 and 6), showing that CPSF-dependent and therefore specific CstF 64 binding was measured. The presence of a 5′ splice site did not affect CstF 64 binding (Fig. 4, lanes 9 and 12). Thus, U1 snRNP does not inhibit cleavage by impairment of CstF, and by implication CPSF, binding to the pre-mRNA.

To test whether binding of CF I_m was inhibited by U1 snRNP, UV crosslinking experiments were performed on the 13S and 13M RNAs incubated with purified cleavage factors. As shown previously (Rüegsegger et al., 1996), all three polypeptides of CF I_m can be UV crosslinked to a pre-mRNA carrying a cleavage/polyadenylation site (Fig. 4B, lanes 1 and 2). Addition of U1 snRNP to the 13S RNA substrate did not lead to a decrease in CF I_m crosslinking (Fig. 4B; lane 3). Thus U1 snRNP did not impair the binding of CF I_m subunits to the RNA.

The remaining well-characterized factor required for the cleavage of some pre-mRNAs is PAP. PAP alone does not bind specifically to RNA but is tethered to poly(A) sites by binding to the 160-kDa subunit of CPSF (Murthy & Manley, 1995). To test PAP assembly into the cleavage complex, pre-mRNAs were incubated in cleavage extracts. Immunoprecipitation was then carried out with a polyclonal antibody directed against PAP and the immunoprecipitated ^{32}P-labeled RNA was visualized after gel electrophoresis. To control the specificity of precipitation, substrate R1 carrying a wild-type or mutant 5′ splice site was precipitated with antibodies directed against PAP in the presence of RNA R2 containing AAUAAA (R2-U) or AAGAAA (R2-G). R2-U was more efficiently precipitated than R1 for unknown reasons (Fig. 5, lane 3), but the mutation of the poly(A) signal in R2 clearly led to preferential precipitation of R1 (Fig. 5, lane 4) showing that PAP was bound specifically to the poly(A) site. Next, the influence of the presence of a downstream 5′ splice site on the recruitment of PAP was tested. No detectable difference be-

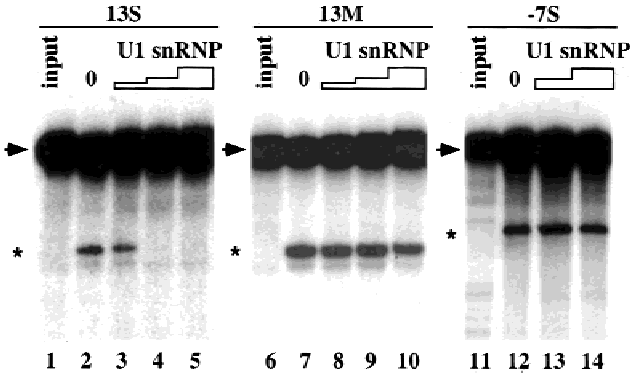

FIGURE 3. Purified U1 snRNP inhibits the cleavage step in a reconstituted cleavage assay. Reconstitution assays were performed as described previously (Rüegsegger et al., 1996). Lanes 1, 6, and 11 were the input substrates. The different pre-mRNAs were incubated in the absence (lanes 1, 2, 6, 7, 11, and 12) or in the presence of increasing amounts (50, 100, or 200 ng) of purified U1 snRNP (lanes 3–5, 8–10, 13, and 14). The precursors are indicated with arrows and the 5′ cleavage product with asterisks.

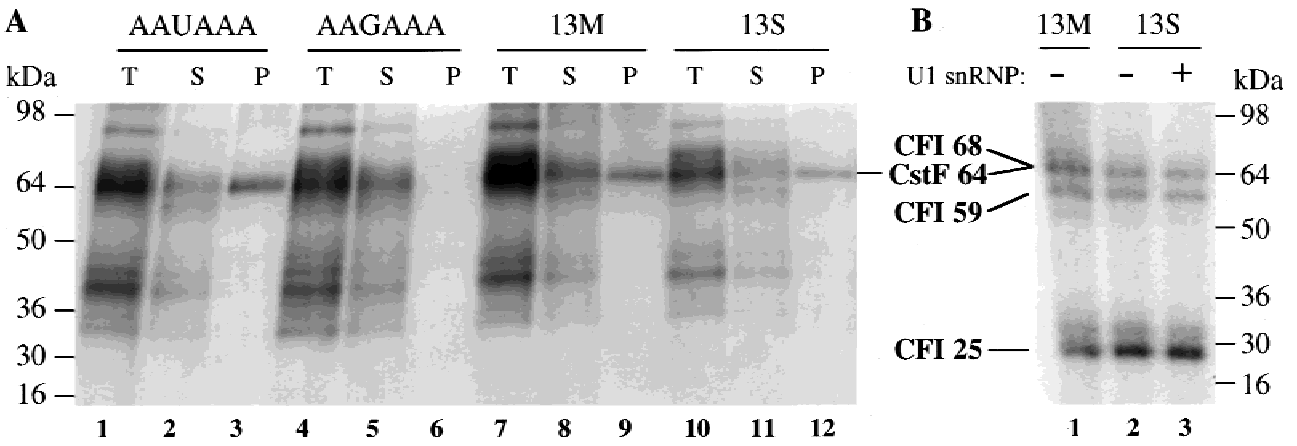

FIGURE 4. U1 snRNP does not impair the binding of CstF 64 or the CF I_m subunits to the RNA. **A**: UV crosslinking/immunoprecipitation experiment with the 64-kDa subunit of CstF. wild-type (AAUAAA) or mutant (AAGAAA) Adenovirus L3 cleavage/polyadenylation site-containing pre-mRNAs (lanes 1–6) or the pre-mRNAs described in Figure 1 (lanes 7–12) were incubated in HeLa cell nuclear extract, crosslinked by UV irradiation, and crosslinked polypeptides were immunoprecipitated using the 3A7 monoclonal antibody directed against the 64-kDa subunit of CstF. Five percent of the crosslinked sample before immunoprecipitation (T) and the full supernatant (S) and pellet (P) fractions after immunoprecipitation were resolved by 10% SDS-PAGE. Protein markers are indicated on the left. **B**: UV crosslinking experiment with CF I_m. The different RNA substrates were incubated with the purified cleavage/polyadenylation components (as in Fig. 3) in the absence (lanes 1–2) or the presence (lane 3) of 200 ng purified U1 snRNP, crosslinked by UV irradiation, and loaded on a 10% SDS-PAGE gel. The position and nature of the crosslinked proteins (64-kDa subunit of CstF and the 68-, 59-, and 25-kDa subunits of CF I_m) are shown on the left, protein markers on the right.

tween immunoprecipitation of the wild-type (R1-13S) and mutant (R1-13M) 5′ splice site-containing RNAs was observed (Fig. 5, compare lanes 5 and 6). Finally, anti-U1A antibody precipitated preferentially the R1-13S RNA containing the 5′ splice site (Fig. 5, lanes 7–10), showing that U1 snRNP was efficiently bound to this RNA but did not affect PAP recruitment, and that U1 snRNP did not bind to the mutant 5′ splice site in the control pre-mRNA.

Taken together, these experiments show that the U1 snRNP-mediated inhibition of cleavage does not occur by impairment of the binding of the CPSF-CstF, CF I_m, or PAP. The binding of all factors involved in cleavage with the exception of CF II_m, for which no specific detection methods are currently available, was therefore unaffected by U1 snRNP.

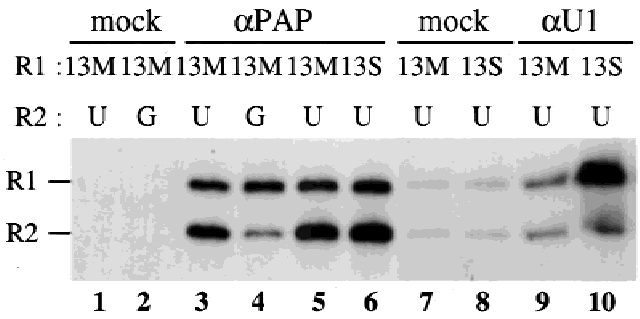

FIGURE 5. Poly(A) polymerase (PAP) recruitment is unaffected by a 5′ splice site. Wild-type (U) or mutant (G) Adenovirus L3 cleavage/polyadenylation site containing pre-mRNA and the substrates described in Figure 1 were incubated in HeLa cell nuclear extract for 2 min under cleavage conditions. Mock immunoprecipitation (lanes 1, 2, 7, and 8) or immunoprecipitation with a polyclonal anti-PAP antibody (lanes 3–6) or a polyclonal anti-U1A antibody (lanes 9–10) was performed. The pellets were fractionated by 10% SDS-PAGE and autoradiographed.

The PAP-U1 70K interaction is not involved in cleavage inhibition

In BPV, U1 snRNP binding to the 5′ splice site upstream of the cleavage/polyadenylation site results in inhibition of polyadenylation via a direct interaction between the U1 70K protein of U1 snRNP and PAP (Gunderson et al., 1998). It was therefore of interest to test the involvement of this interaction on U1 snRNP-mediated cleavage inhibition. The role of the domain of PAP that interacts both with the U1 70K protein and with a dimer of the U1A protein was investigated with a mutant form of PAP from which the U1 70K interaction region was deleted (PAP Δ695–739; Gunderson et al., 1998). In the reconstituted cleavage assay this mutation had no effect on the ability of U1 snRNP to mediate cleavage inhibition on the 13S RNA substrate, where the 5′ splice site is located 13 nt downstream of the cleavage site (Fig. 6B; lanes 2–4). The possibility of a contamination by wild-type PAP in the purified cleavage factors used in the assay was ruled out by demonstrating that cleavage did not occur without additional PAP (Fig. 6A, compare lanes 2 and 3). Thus, PAP-U1 70K interaction is not necessary for U1 snRNP-mediated inhibition of cleavage.

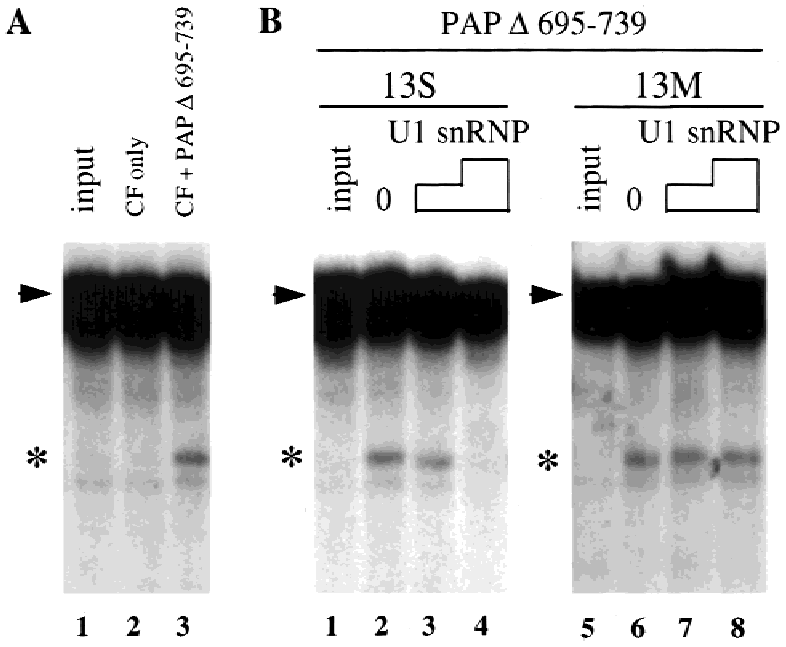

FIGURE 6. The PAP-U1 70K interaction is not involved in U1 snRNP-mediated inhibition of cleavage. Reconstituted cleavage assays in the presence of purified cleavage/polyadenylation components (Rüegsegger et al., 1996). Cleavage assays are as in Figure 3, except that a mutant of PAP (PAP Δ695–739) produced in *E. coli* was used in place of wild-type PAP. **A**: The 13M pre-mRNA was incubated with all the purified cleavage factors (CF) necessary for the reconstituted cleavage assay without (lane 2) or with (lane 3) PAP Δ695–739. Lane 1: input substrate. **B**: The 13S or 13M pre-mRNAs were incubated with cleavage factors in the absence (lanes 1, 2, 5, and 6) or in the presence of increasing amounts (100 or 200 ng) of purified U1 snRNP (lanes 3, 4, 7, and 8). Lanes 1 and 5: input substrates. The precursors are indicated with arrows and the 5′ cleavage product with asterisks.

The U1A and U1 70K proteins are not sufficient for U1 snRNP-mediated inhibition of cleavage

Although not involved in an interaction with PAP, the U1 70K or the U1A proteins could interact with other cleavage factors to mediate the inhibition of cleavage. To test this possibility, we performed reconstituted cleavage reactions with a partly reconstituted U1 snRNP consisting of an in vitro-transcribed modified U1 snRNA and recombinant U1A and U1 70K proteins (Gunderson et al., 1998). Because of the poor efficiency of binding of U1 snRNA to a 5′ splice site-containing RNA, a modified U1 snRNA is used (U1 snRNA*) with an extended 5′ end that allows “prosthetic” base pairing to an extended 5′ splice site (Gunderson et al., 1998). The 68S pre-mRNA substrate RNA (Fig. 1) contains this extended complementarity to U1 snRNA*. A U1A-U1 snRNA*, a U1 70K-U1 snRNA*, and a U1A-U1 70K-U1 snRNA* complex were preassembled (Gunderson et al., 1998) and each was added separately to the 68S pre-mRNA substrate. Purified cleavage components were then added to the different complexes. No inhibition of cleavage was observed in any case (Fig. 7). Native gel electrophoresis confirmed that U1 snRNA*-13S pre-mRNA complexes were correctly assembled in each case and addition of the U1 70K-containing RNPs inhibited polyadenylation of BPV pre-mRNA as previously shown (data not shown; Gunderson et al., 1998). Therefore, unlike previous cases of 3′-end formation inhibition, the U1A and U1 70K proteins are not sufficient for the U1 snRNP-mediated inhibition of cleavage at an upstream site. This indicates that the molecular interactions involved in cleavage inhibition and in polyadenylation inhibition by U1 snRNP are different.

DISCUSSION

The results presented in this study reveal that U1 snRNP can act in two mechanistically quite diverse ways to

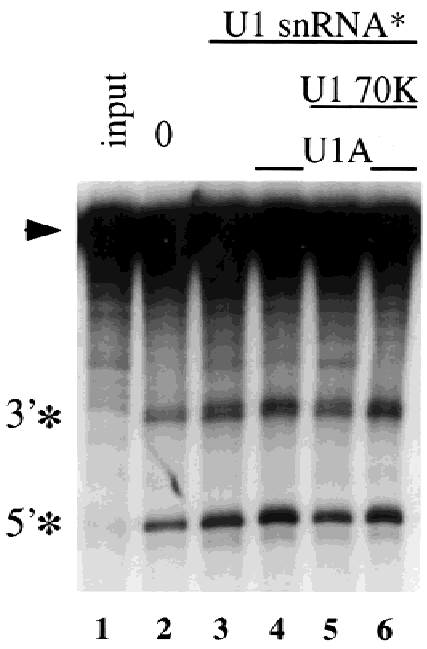

FIGURE 7. The U1A and U1 70K proteins of U1 snRNP are not sufficient for U1 snRNP-mediated cleavage inhibition. Reconstituted cleavage assays with the 68S pre-mRNA and with reconstituted U1 snRNP containing a modified U1 snRNA (U1 snRNA*) which forms an extended base pairing interaction with the 68S pre-mRNA 5′ splice site region. U1 snRNA* was added either alone (lane 3) or after preassembly with recombinant U1A (lane 4), U1 70K (lane 5), or both U1A and U1 70K proteins (lane 6). Lane 1 is the input substrate. Lane 2 contains all the cleavage components but no reconstituted U1 snRNP. *5′ and *3′ are the 5′ and 3′ cleavage products, respectively. The precursor is indicated with an arrow.

inhibit 3′-end processing of pre-mRNAs. In vitro cleavage reactions in HeLa cell nuclear extract and reconstituted cleavage assays were used to show that U1 snRNP binding to a 5′ splice site located downstream of the signals directing cleavage and polyadenylation inhibits the first step of the reaction, 3′-end cleavage. Previous work had shown that binding of U1 snRNP to a 5′ splice site upstream of the same signals, in contrast, inhibited the polyadenylation step of 3′-end processing (Gunderson et al., 1998). Whereas the latter reaction required a specific interaction between the U1 70K protein of U1 snRNP and poly(A) polymerase, this interaction is not sufficient for the inhibition of cleavage by U1 snRNP. Because removal of the U1 70K interaction domain from PAP does not affect the inhibition of cleavage by U1 snRNP, it further seems that the previously characterized interactions between U1 70K, U1A, and PAP are not involved in cleavage inhibition.

Mechanism of cleavage inhibition by U1 snRNP

The detailed mechanism by which U1 snRNP inhibits cleavage remains to be determined. Here, the main difficulty is that although many, but not all (see Introduction), of the components required for 3′-end cleavage have been identified, not much is known about the mechanism by which cleavage occurs. Cleavage requires a large number of proteins that assemble into a complex on the cleavage/polyadenylation site. An understanding of how the different protein partners are arranged at the cleavage/polyadenylation site is emerging, but is as yet incomplete. In addition, the protein or complex of proteins that constitutes the active site of the required endonuclease activity is not yet identified.

In principle, U1 snRNP could either block one or more steps required for the correct assembly of the cleavage/polyadenylation machinery or it could inhibit an enzymatic activity required for cleavage. The data presented here indicate that the assembly processes required for binding of CPSF, CstF, CF I_m, and PAP to the pre-mRNA do not seem to be affected by U1 snRNP binding. The inhibitory mechanism is therefore probably not a simple steric hindrance and likely involves direct interaction between U1 snRNP and one or more of the cleavage factors.

Three separate interactions between U1 snRNP components and the 3′-end formation machinery have previously been described. The U1A protein, independently of U1 snRNP, is able to interact with the 160-kDa subunit of CPSF, and this interaction has a stimulatory effect on polyadenylation (Lutz et al., 1996). Both the U1A protein, this time as a dimer, and the U1 70K protein as a monomer can interact with the extreme C-terminal segment of PAP (Gunderson et al., 1997, 1998). Both of these interactions inhibit PAP activity and thus block polyadenylation. The inhibition of cleavage by U1 snRNP does not seem to involve any of the known interactions. Moreover, although inhibition by a direct interaction between U1 70K and the 68-kDa subunit of CF I_m via their SR-like domains seemed plausible (Theissen et al., 1986; Rüegsegger et al., 1998), this interaction is certainly not sufficient for inhibition (see Fig. 7), as neither U1 70K or U1A alone or in combination is sufficient to mediate the inhibitory effect on cleavage. Thus, the mechanism of inhibition could involve the other U1 snRNP-specific protein, namely the U1C protein or one of the Sm core proteins (Lührmann et al., 1990). Alternatively, a complex aspect of U1 snRNP structure involving more than one of its components could be required.

The inhibitory effect of U1 snRNP on cleavage is expected to be general because it was observed with different synthetic RNA substrates containing 5′ splice site insertions at various positions and in two different sequence contexts. The first indication of an inhibitory effect of U1 snRNP on cleavage, strongly predicted to involve a similar mechanism to that described here, was observed in vivo with a different cleavage/polyadenylation site, that present in the 5′ LTR of HIV-1, and in that case inhibition was also observed in various contexts (Ashe et al., 1995, 1997). A requirement for a certain distance between the sites of 3′-end formation and U1 snRNP binding or for a particular RNA structure in the pre-mRNA could exist, however. Increasing the distance between the 5′ splice site and the cleavage site in HIV-1 pre-mRNA abolished the inhibitory effect in vivo (Ashe et al., 1997). This would suggest that also in vivo, the U1 snRNP has to interact directly with the cleavage/polyadenylation machinery.

The influence of relative position on the inhibitory mechanism

When bound upstream of the cleavage/polyadenylation site, U1 snRNP does not inhibit cleavage but instead, through binding of the U1 70K protein of U1 snRNP to PAP, inhibits polyadenylation (Gunderson et al., 1998). Establishing why the snRNP is not capable of inhibiting cleavage from this position will obviously require a much more detailed knowledge of the structure formed by the cleavage and polyadenylation factors when they bind to an RNA. However, the two inhibitory mechanisms have quite different requirements, indicating that they probably evolved separately. U1 snRNP bound to a site downstream of the point of 3′ cleavage may be removed from the template for poly(A) addition immediately after 3′ cleavage, and thus be incapable of participating in the inhibition of polyadenylation even if the U1 70K of this snRNP had been in contact with PAP prior to the cleavage event. The consequences of the two types of inhibition are also quite different. Inhibition of the first step allows the accumulation of pre-mRNA transcripts that read through a potential site of 3′-end

formation. This is used by HIV-1 to ensure the production of full length transcripts of its genome rather than short RNAs truncated in the 5′ LTR. Clearly, the same mechanism could be used to control the use of 3′-end formation sites in cellular pre-mRNAs, perhaps in a regulated fashion.

An interesting further possibility is that this same mechanism could operate generally in pre-mRNAs to help prevent 3′-end processing occurring in the 5′ terminal exon or in internal exons rather than in the 3′-terminal exon. An AAUAAA sequence will occur every 4 kb on average, and this sequence can frequently be found elsewhere than in 3′ terminal exons. How many of these AAUAAA signals might potentially be recognized by the 3′-end formation apparatus is unclear, mainly because the poor conservation of the downstream elements of sites of 3′-end formation makes them difficult to predict from DNA sequence data. A mechanism may therefore exist to inhibit the use of cryptic cleavage/polyadenylation sites located in upstream exons. As cleavage/polyadenylation sites present in these exons will always be located upstream of a 5′ splice site, it could be that U1 snRNP binding inhibits the use of any potential sites. Only cleavage/polyadenylation sites present in 3′ terminal exons that are not upstream of a nearby 5′ splice site would then be used.

The consequences of the second mode of inhibition, that is, of U1 snRNP inhibiting the second step of 3′-end formation, are quite different. Here, cleaved but nonadenylated RNAs are produced. These will presumably be unstable. Inhibiting polyadenylation but permitting cleavage can therefore be used as an on/off switch for particular genes, as exemplified both by the U1A autoregulatory loop, where excess U1A protein switches off the production of U1A mRNA, and the BPV case where U1 snRNP prevents accumulation of late viral mRNAs at early times in infection. The quite different logic behind the two forms of regulation of 3′-end formation by U1 snRNP further supports the hypothesis that they probably evolved entirely separately.

Taken together, our observations underline the notion that the splicing and the cleavage and polyadenylation processing machineries assembled on a pre-mRNA influence each other. With the in vitro reconstituted system described here and further advances in understanding the mechanism of 3′ cleavage, it will be possible to address the detailed mechanism of cleavage inhibition by U1 snRNP.

MATERIALS AND METHODS

Plasmid constructions

Plasmids were constructed by standard cloning procedures. They are all pSP64 derivatives. pSP-U (or AdL3) is the original plasmid described in Boelens et al. (1993). It contains nt 22293–22600 of the Adenovirus L3 late region that includes the cleavage/polyadenylation site. The first 60 nt are derived from the pSP64 vector. pSP-G was derived from pSP-U and has an AAUAAA-to-AAGAAA mutation in the Adenovirus L3 cleavage/polyadenylation signal. pSP-7S was constructed by inserting a wild-type 5′ splice site sequence 18 nt upstream of the cleavage site (Gunderson et al., 1998). pSP-7M was derived from pSP-7S by an AAGGTAAGT-to-AAG**CA**AA**C**T mutation in the 5′ splice site sequence. The critical sequence region in -7S is GAATTC**CAGGTAAGT**AGTTATC**AAUAAA** and in -7M GAATTC**CAGCAAACT**AGTTATC**AAUAAA**. pSP13S and pSP52S were constructed by insertion of the 5′ splice site sequence 13 or 52 nt downstream of the cleavage site. The mutant 5′ splice site was incorporated into the pSP13M and pSP52M plasmids. pSP68S and pSP68M were constructed by insertion of a sequence (CGTAAATGTAAAACAGAAGGTAAGT) corresponding to a 5′ splice site with an extended potential base pairing with the prosthetic U1 snRNA* described in Gunderson et al. (1998) or of a matched sequence mutated at the 5′ splice site (CGTAAATGTAAAACAGAAG**CA**AA**C**T).

Expression and purification of recombinant proteins

The wild-type and mutant (Δ695–739) PAP proteins with a carboxy-terminal histidine tag were produced in *Escherichia coli* and purified by Ni^{2+}-NTA chromatography (Gunderson et al., 1997). The human U1A protein was purified from *E. coli* (Gunderson et al., 1994). The C-terminally His-tagged human 70K protein was expressed in Sf9 cells using the Bac-to-Bac expression kit (Gibco-BRL) and purified as described in Gunderson et al. (1998).

Purification of U1 snRNP

U1 snRNP was purified using an adaptation of the procedure described by Bach et al. (1990). Thirty milliliters of buffer 1 (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) were added to 20 mL of HeLa nuclear extract (200 mg protein). The material was filtered (0.45 μM) and slowly (3 mL/h) loaded onto a 1-mL TMG cap antibody column (Oncogene Science) that had been equilibrated in buffer B (buffer A + 10% glycerol). The unbound material was collected and the column washed with buffer B. The material bound to the TMG column (total snRNP particles, i.e., U1, U2, U4, U5, U6) was eluted by soaking the column overnight in buffer B + 15 mM 7-methylguanosine (Sigma). The eluate was applied to a MonoQ column using running buffer C (20 mM Tris, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT). A linear gradient from 0 to 600 mM KCl resulted in highly purified U1 snRNP fractions eluting around 350 mM KCl.

Cleavage/polyadenylation reactions in HeLa nuclear extracts

Nuclear extracts were prepared from HeLa cells by the procedure of Dignam et al. (1983). Substrate RNAs were transcribed by SP6 RNA polymerase and m^7GpppG capped. In vitro reactions in HeLa cell nuclear extracts were as de-

scribed (Boelens et al., 1993). Oligonucleotide-mediated RNAse H digestion of U1 snRNP and U2 snRNP was achieved by addition of DNA oligonucleotides complementary to nt 1–14 of U1 snRNA or 40–70 of U2 snRNA to HeLa nuclear extract (Krämer & Keller, 1990). After a 15-min incubation, the labeled substrate was then added and the reaction was continued for 120 min. Reconstituted cleavage reactions were performed as described in Rüegsegger et al. (1996) with additions as described in the text.

UV crosslinking/immunoprecipitation

HeLa cell nuclear extract was incubated for 5 min with ^{32}P-labeled RNAs under cleavage conditions. The reaction mixtures were then irradiated with UV light (254 nm) in a Stratalinker (Stratagene) at 0.4 J/cm^2 on ice at a distance of 10 cm from the lamps. Fifty units of RNAse T1 were then added and the reaction mixtures were incubated for 30 min at 37 °C. SDS gel loading buffer was added and the samples were boiled for 2 min before they were fractionated on a 10% SDS-polyacrylamide gel. For immunoprecipitation of UV crosslinked proteins, 20 μL of the RNAse T1-treated samples were diluted in 200 μL of IP2 buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05% NP-40), precleared, and mixed with 7 μL of anti-CstF 64 monoclonal antibody (MacDonald et al., 1994). The mixtures were allowed to rotate 1 h at 4 °C. Protein G beads were added to the mixtures and incubations continued for 1 h at 4 °C. After extensive washing of the beads, the bound proteins were eluted in SDS-loading buffer.

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