Interaction between the U1 snRNP–A protein and the 160-kD subunit of cleavage–polyadenylation specificity factor increases polyadenylation efficiency in vitro

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We have previously shown that the U1 snRNP–A protein (U1A) interacts with elements in the SV40 late polyadenylation signal and that this association increases polyadenylation efficiency. It was postulated that this interaction occurs to facilitate protein–protein association between components of the U1 snRNP and proteins of the polyadenylation complex. We have now used GST fusion protein experiments, coimmunoprecipitations and Far Western blot analyses to demonstrate direct binding between U1A and the 160-kD subunit of cleavage–polyadenylation specificity factor (CPSF). In addition, Western blot analyses of fractions from various stages of CPSF purification indicated that U1A copurified with CPSF to a point but could be separated in the highly purified fractions. These data suggest that U1A protein is not an integral component of CPSF but may be able to interact and affect its activity. In this regard, the addition of purified, recombinant U1A to polyadenylation reactions containing CPSF, poly(A) polymerase, and a precleaved RNA substrate resulted in concentration-dependent increases in both the level of polyadenylation and poly(A) tail length. In agreement with the increase in polyadenylation efficiency caused by U1A, recombinant U1A stabilized the interaction of CPSF with the AAUAAA-containing substrate RNA in electrophoretic mobility shift experiments. These findings suggest that, in addition to its function in splicing, U1A plays a more global role in RNA processing through effects on polyadenylation.

[Key Words: U1 snRNP; polyadenylation; cleavage and polyadenylation specificity factor]

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Formation of mature messenger RNA (mRNA) requires precise RNA processing, including splicing and polyadenylation (for review, see Manley 1988; 1995; Luhrmann et al. 1990; Wickens 1990; Wahle and Keller 1992; Moore et al. 1993; Sachs and Wahle 1993). Splicing involves removal of intronic sequences and ligation of exons by a complex set of small nuclear ribonucleoprotein particles (snRNPs) and other factors known collectively as the spliceosome. Polyadenylation is the process by which the 3' end is formed through specific endonucleolytic cleavage of the precursor RNA and the addition of ~250 adenosine residues. Both in vitro (Niwa et al. 1990; Niwa and Berget 1991) and in vivo (Chiou et al. 1991; Nesic et al. 1993; Nesic and Maquat 1994) experiments

have suggested that the processes of splicing and polyadenylation might be functionally linked. This proposal has been supported by our previous report that the U1 snRNP–A protein (U1A) interacts with elements in the SV40 late polyadenylation signal and that these interactions increase polyadenylation efficiency (Lutz and Alwine 1994). These data support the exon definition model of Berget and co-workers (for review, see Berget 1995), which suggests that components of both the spliceosome and the polyadenylation complex may interact to define the last exon and affect the efficiencies of polyadenylation and last intron removal.

There are currently five established mammalian factors comprising the complex that cleaves and polyadenylates substrate RNAs: cleavage–polyadenylation specificity factor (CPSF), cleavage stimulatory factor (CstF), poly(A) polymerase (PAP), and cleavage factors I and II (CFI and CFII) (for review, see Manley 1995). PAP is re-

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sponsible for the addition of the adenosine residues to the cleaved product (Ryner et al. 1989a; Bardwell et al. 1990; Raabe et al. 1991, 1994; Wahle et al. 1991). CFI and CFII have not yet been well characterized but appear to be proteins of ~110 and 135 kD, respectively, that are likely responsible for the cleavage of the pre-mRNA (Takagaki et al. 1989). CstF consists of three subunits of 50, 64, and 77 kD (Takagaki et al. 1990, 1992; Gilmartin and Nevins 1991; Takagaki and Manley 1992, 1994) and interacts with CPSF to help specify the polyadenylation site. CPSF has three subunits, 160, 100, and 70 kD, and perhaps an additional subunit of ~30 kD (Bienroth et al. 1991; Murthy and Manley 1992). cDNAs encoding both the 100-kD (Jenny et al. 1994) and the 160-kD (Murthy and Manley 1995; Jenny and Keller 1995) subunits of CPSF have recently been isolated. The 160-kD subunit of CPSF appears to be responsible, at least in part, for recognition of the AAUAAA critical to the formation of the cleavage and polyadenylation complex (Keller et al. 1991; Murthy and Manley 1995).

Efficient utilization of a polyadenylation signal requires recognition of not only the AAUAAA but also specific elements within the substrate RNA both upstream and downstream of the AAUAAA. Both downstream elements (DSEs) and upstream elements (USEs) affect the efficiency of utilization of an AAUAAA in polyadenylation; their position relative to the AAUAAA appears to be critical for the effect (Bar-Shira et al. 1991; Gilmartin et al. 1992; Schek et al. 1992; Chou et al. 1994). DSEs have been described in the polyadenylation signals of many viral and cellular genes. These elements tend to be GU- or U-rich and are located between 14 and 70 nucleotides downstream from the AAUAAA (Gil and Proudfoot 1984, 1987; McDevitt et al. 1984, 1986; Sadofsky and Alwine 1984; Cole and Stacy 1985; Conway and Wickens 1985; Sadofsky et al. 1985; Zhang and Cole 1987; Wilusz et al. 1988; Zarkower and Wickens 1988; Ryner et al. 1989b; Wilusz and Shenk 1990). It is generally felt that DSEs are a standard feature of mammalian polyadenylation signals. This is supported by the observation that the 64-kD subunit of CstF interacts with the DSE (MacDonald et al. 1994; Y. Takagaki and J. Manley, in prep.). USEs have been described in many viral systems at distances of 10–35 nucleotides upstream of the AAUAAA hexamer (Carswell and Alwine 1989; DeZazzo and Imperiale 1989; Russnak and Ganem 1990; Brown et al. 1991; DeZazzo et al. 1991; Russnak 1991; Sanfacon et al. 1991; Valsamakis et al. 1991, 1992; Gilmartin et al. 1992; Schek et al. 1992). Previously, we have described the USE motifs found in SV40 late mRNA (Carswell and Alwine 1989; Schek et al. 1992) and have shown that the U1A protein utilizes these motifs to interact with the RNA (Lutz and Alwine 1994). This interaction significantly affects the efficiency of utilization of the SV40 late polyadenylation signal in vitro and has led us to suggest that the interaction of the U1A protein with the USE motifs, close to the AAUAAA, could increase the possibility of interactions between the U1 snRNP and the cleavage and polyadenylation complex associated with the AAUAAA. Hence,

this putative protein–protein interaction would provide a molecular mechanism not only for the linkage observed previously between splicing and polyadenylation but also the definition of last exon.

In this communication we provide in vivo and in vitro evidence that U1A protein can directly interact with the 160-kD subunit of CPSF. This interaction correlates with in vitro studies showing that U1A can both increase CPSF-dependent polyadenylation and enhance the binding of CPSF to an AAUAAA-containing substrate RNA.

Results

The data discussed above suggest that interactions likely occur between the polyadenylation complex and snRNP components to affect the efficiency of the polyadenylation reaction. To establish that such interactions exist, we first performed a number of experiments to examine possible associations between specific proteins in the cleavage and polyadenylation complex and in the U1 snRNP.

Examination of purified fractions of CPSF for the presence of the U1 snRNP proteins

To test the possibility that U1A protein might associate with CPSF, crude HeLa cell nuclear extract as well as samples of CPSF taken from various stages of purification of calf thymus CPSF (Murthy and Manley 1992) were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed by Western blotting. The blot was probed simultaneously with two polyclonal antibodies, one specific for the 160-kD CPSF subunit and the other for U1A protein (Fig. 1A). The position of U1A protein was readily detected in the crude HeLa cell nuclear extract (lane 1); however, no 160-kD protein was visualized. This is attributable to (1) the difference in abundance of these two proteins in crude extracts and (2) a significantly lower sensitivity of the anti-160 antibody in relation to the anti-U1A antibody (data not shown). Both proteins were detected in the heparin–agarose fraction (lane 2). The differences in intensity of the two bands cannot be considered quantitatively because of the differences in the sensitivity of the two antibodies. The heparin–agarose fraction comes from the middle of the purification scheme used: nuclear extract; DEAE–Sephacrose; ammonium sulfate; phosphocellulose; Superose 6; heparin–agarose; Mono Q; poly(U)–cellulose; spermine–agarose; phenyl–Superose; and glycerol gradient (Murthy and Manley 1992). In addition, both proteins were detected in the Mono Q fraction (data not shown). However, in the poly(U) and spermine–agarose fractions the 160-kD protein was readily detectable, whereas U1A protein appears to have been separated from CPSF (lanes 3,4).

The same blot was then stripped and reprobed with a monoclonal antibody specific for the B/B' proteins of the Sm complex of proteins common to all snRNPs (Lührmann et al. 1990). This would indicate whether U1A

Figure 1. Western blots performed to detect U1A, 160-kD protein of CPSF and Sm antigen in HeLa nuclear extract and partially purified CPSF fractions. (Lanes 1) Nuclear extract prepared from HeLa cells; (lanes 2) calf thymus CPSF fraction from heparin-agarose; (lanes 3), CPSF fraction from poly(U)-cellulose; (lanes 4) CPSF fraction from spermine agarose. Each sample of purified CPSF fractions contained approximately equal CPSF activity (Murthy and Manley 1992). (A) The result of probing the blot simultaneously with anti-U1A and anti-160-kD protein rabbit antibodies. The blot was then stripped and reprobed with an anti-Sm mouse monoclonal antibody; these results are shown in B. Molecular weight markers are indicated at left.

alone or U1A as part of a snRNP was copurifying with CPSF through the heparin-agarose steps. Figure 1B shows that whereas SmB/B' proteins were detected in the HeLa nuclear extract (lane 1), they were not present in any of the purified CPSF fractions tested, especially the heparin-agarose fraction that contained U1A. In ad-

dition, examination of similar U1A-containing fractions for the presence of the U1 snRNP-70-kD protein indicated that this protein was also absent (data not shown). These data suggest that the U1A protein may be present in these fractions free of most other snRNP components. Published results have shown that small but detectable amounts of U1 RNA are present in the heparin-agarose CPSF fraction, although not in later fractions (Murthy and Manley 1992). Therefore, we cannot definitively determine whether the U1A protein detected was free or bound to U1 RNA from which other snRNP proteins, that is, the Sm antigens and the 70-kD protein, are absent.

These data suggest that a labile association may occur *in vivo* between U1A and a component of CPSF. Apart from its significance in providing a possible link between splicing and polyadenylation, this finding is intriguing in light of the fact that some preparations of purified CPSF contain a fourth, RNA-binding subunit of ~30 kD (e.g., Jenny et al. 1994), whereas others lack this component (Murthy and Manley 1992; Gilmartin et al. 1995). Our data raise the possibility that this 30-kD polypeptide may be U1A, which would be maintained bound to CPSF in some purification procedures and removed in others. However, future work is required to determine the relationship, if any, between these two proteins. The following experiments were done to establish that there is indeed an interaction between U1A and CPSF.

[³⁵S]Met-labeled 160-kD protein interacts with U1A by Far Western analysis

To further examine the possibility of an association between U1A and CPSF we employed the Far Western protein-blotting technique (Lee et al. 1991) in which immobilized, renatured target proteins on the blot are detected using a specific labeled protein probe. In Figure 2A purified, bacterially expressed, histidine (His)-tagged U1A (~34 kD) was displayed on SDS-polyacrylamide gels and transferred to nitrocellulose. Figure 2B shows Coomassie blue-stained SDS-PAGE analysis of the purified His-tagged U1A used in this and other experiments described below.

Recent experiments have indicated that the 160-kD subunit of CPSF plays a key role in CPSF function, interacting with several components of the polyadenylation complex (Murthy and Manley 1995). Therefore, this protein seemed a logical candidate to mediate the interaction of CPSF with U1A. Identical protein blots of purified His-tagged U1A were probed as follows: (1) Western blot analysis was performed on one blot using a monoclonal antibody to U1A (lane αU1A) to determine the position of His-tagged U1A protein; (2) an identical blot was probed with an *in vitro* transcribed-translated ³⁵S-labeled 160-kD subunit of CPSF (lane 160, Murthy and Manley 1995); (3) as a control an identical blot was probed with an *in vitro* transcribed/translated ³⁵S-labeled immediate early protein encoded by the human cytomegalovirus (IEP72; IE1_{491aa}; ppUL123; Stinski 1990; lane IEP72). The ³⁵S labeled 160-kD protein bound

to the purified His-tagged U1A, whereas the control protein (IEP72) did not.

To obtain evidence that the observed interaction between the 160-kD subunit and U1A was specific, the crude bacterial extract from which the His-tagged U1A was purified was subjected to Far Western blotting and probed with in vitro-transcribed/translated ^{35}S -labeled 160-kD subunit of CPSF (Fig. 2C, lane 160). The adjacent lane (stained) shows the Coomassie blue staining of an SDS-polyacrylamide gel containing the same amount of the bacterial extract. It can be seen that the labeled 160-kD protein interacted specifically with the U1A protein but not with bacterial proteins, suggesting that the 160-kD protein has little nonspecific binding.

The above data support a specific and direct protein-protein interaction between the U1A protein and the 160-kD component of CPSF. The following experiments provide additional support for such an interaction.

Examination of binding between GST-U1A and CPSF 160-kD protein

To address the 160-kD-U1A interaction by another method, the glutathione binding moiety from glutathione *S*-transferase (GST) was fused to the amino terminus of the full-length U1A protein (GST-U1A). In addition, the GST moiety was fused to the separated amino-terminal (amino acids 1–134; GST-U1Aamino) and carboxy-terminal (amino acids 135–282; GST-U1Acarb) halves of the protein which, respectively, contain RNA recognition motif 1 and 2 (RRM1 and RRM2; Sillickens et al. 1987; Scherly et al. 1989; Lutz-Freyermuth et al. 1990). These fusion proteins were expressed in *Escherichia coli* and purified on glutathione-agarose. Figure 3A shows a silver-stained SDS-PAGE analysis of the various purified fusion proteins.

GST-U1A and the GST moiety alone were tested for binding to in vitro-transcribed/translated ^{35}S Met-labeled 160-kD protein. The complexes bound to gluta-

thione-agarose were washed extensively in increasing NaCl concentrations, and the remaining bound proteins were then fractionated by SDS-PAGE. Figure 3B shows that the GST-U1A fusion protein bound the 160-kD protein, whereas the GST moiety alone retained only background levels of the 160-kD protein. Under normal binding and washing conditions of 100 mM NaCl we routinely detected between 10% and 15% of the input 160-kD protein bound specifically to GST-U1A. Signif-

Figure 2. Far Western protein blotting for the detection of interaction between U1A and 160-kD protein of CPSF. (A) Purified recombinant His-tagged U1A protein was separated on a 12.5% SDS-polyacrylamide gel, electroblotted to nitrocellulose and prepared for Western and Far Western analyses (see Materials and methods). The first blot (lane αU1A) was subjected to Western analysis using a mouse monoclonal antibody specific for the U1A protein as the probe. The second blot (lane 160) was subjected to Far Western analysis using ^{35}S Met-labeled 160-kD protein as a probe. The third blot (lane IEP72) was subjected to Far Western analysis using ^{35}S Met-labeled HCMV IEP72 protein. (B) A sample of the purified, recombinant His-tagged U1A protein used in this and other experiments was subjected to SDS-PAGE analysis and stained with Coomassie blue. Molecular weight markers are indicated at left. (C) Samples of the crude bacterial extract from which the His-tagged U1A was purified were separated by SDS-PAGE as described above. One lane was subjected to Far Western analysis using ^{35}S Met-labeled 160-kD protein as a probe (lane 160). The other lane (stained) was stained with Coomassie blue.

Figure 3. GST-U1A interacts with the 160-kD component of CPSF. (A) A silver-stained gel of proteins fused to the GST moiety. Each protein was induced in *E. coli* and purified on glutathione-agarose. (GST) GST moiety alone; (GST-U1A) GST fusion to U1A protein; (GST-U1Aamino) GST fusion protein with the amino-terminal half of the U1A protein (amino acids 1–134); (GST-U1Acarb) GST fusion protein with the carboxy-terminal half of the U1A protein (amino acids 135–282). (B) Binding of [35 S]Met-labeled in vitro-transcribed/translated 160-kD protein to GST-U1A and to the GST moiety alone. Bound proteins were washed in increasing NaCl concentrations (mM) as indicated across the top. (Left lanes) Binding of 160-kD protein to GST-U1A; (right lanes) binding of 160-kD protein to GST moiety alone. (C) Binding of [35 S]Met-labeled 160-kD protein to the GST fusions to the amino- and carboxy-terminal halves of U1A.

icant binding was still detected under washing conditions of 500 mM NaCl but eliminated by 1 M NaCl; the partial salt resistance may indicate some nonionic characteristics of the interaction. Figure 3C shows that the 160-kD protein bound to GST-U1Aamino and not to GST-U1Acarb, indicating that the amino-terminal half of the U1A protein contains sufficient structure to allow 160-kD protein binding.

Because both U1A and the 160-kD subunit of CPSF are RNA-binding proteins (Lutz-Freyermuth et al. 1990; Murthy and Manley 1995) it is possible that the apparent interaction of the 160-kD protein with GST-U1A resulted from tethering of the two proteins by RNA present in the reticulocyte lysate or bound to the bacterially produced protein. To test this possibility the binding reactions were treated with RNases A and T1 (see Materials and methods). Lanes 7 and 8 of Figure 4 show that the GST-U1A/160-kD complex was stable to RNase treatment, indicating that the association between the two proteins was attributable to protein-protein interactions. Lanes 3 and 4 demonstrate that an apparent association of U1A protein with itself [GST-U1A protein with in vitro-transcribed/translated [35 S]Met-labeled U1A] was sensitive to RNase treatment. This indicates the importance of this test in verifying the nature of interactions between RNA-binding proteins in this type of analysis.

Analysis of the interaction between U1A protein and the 160-kD protein by coimmunoprecipitation

To verify the binding indicated by the Far Western and

GST fusion protein experiments, U1A protein and 160-kD protein were prepared by in vitro transcription and translation and labeled with [35 S]Met (Fig. 5A, lanes 6 and 7, which represent 10% of the input of each protein used in the precipitation experiments). The 160-kD protein produced for this experiment contained a phage T7 gene 10 (g10) epitope tag that was utilized to analyze coimmunoprecipitation of the two proteins. Approximately equimolar amounts of the two proteins were incubated separately or together and immunoprecipitated using an anti-g10 antibody or a preimmune serum as described in Materials and methods. The data in Figure 5A indicate clearly that the anti-g10 antibody specifically precipitated the tagged 160-kD protein (lane 2) and not U1A (lane 3), whereas preimmune serum recognized neither protein (lanes 4 and 5). However, when both U1A protein and 160-kD protein were mixed, the two proteins were coprecipitated. This result further supports the existence of a direct association between the 160-kD protein of CPSF and the U1A protein. It should be noted that although repeats of the coimmunoprecipitation experiment always indicated coprecipitation of U1A with 160 kD protein, the quantity of U1A can be lower than that shown in Figure 5A, indicating that the interaction may be relatively unstable. This agrees with the data in Figure 1 which suggested that a labile association occurs in vivo between U1A and CPSF.

To confirm that the coimmunoprecipitation of in vitro-produced proteins mimicked an in vivo association, we determined whether the 160-kD subunit of CPSF and U1A protein could be coprecipitated from a HeLa cell nuclear extract. In the experiment shown in

Figure 4. RNase treatment of the complex of GST-U1A and 160-kD protein. The GST moiety (GST) or GST-U1A was incubated with [35 S]Met-labeled U1A (lanes 1–4) or 160-kD (lanes 5–8). After binding and washing of bound proteins one set of samples was incubated with 22 μ g of RNase A and 1×10^4 units of RNase T1 for 30 min at room temperature (RNase +, lanes 2,4,6,8) and then prepared for SDS-PAGE analysis. An identical set of mock-digested samples was prepared and analyzed by SDS-PAGE (RNase –, lanes 1,3,5,7). The input lanes 9 and 10 represent 5% of the amount of labeled U1A or 160-kD protein added to the binding reactions.

Figure 5, B and C, HeLa nuclear extracts were precipitated with preimmune serum, polyclonal anti-U1A, or polyclonal anti-160. The immunoprecipitates were then subjected to Western blot analysis using monoclonal anti-U1A antibody as the probe. In Figure 5B the position of the U1A protein was clearly indicated in the anti-U1A precipitate. In addition, a specific band of U1A was detected in the precipitate using anti-160 antibodies, whereas little U1A was precipitated by the preimmune antibodies. Figure 5C shows a repeat of this experiment; the results are the same except that the small amount of U1A in the preimmune sample has been eliminated by additional washing. The weak U1A protein signal detected in the anti-160 precipitates was not unexpected, as it is likely that only a small fraction of U1A protein is stably associated with CPSF. In separate experiments (not shown) we utilized purified His-tagged U1A in immunoprecipitation experiments with the anti-160 antibodies. These data showed that the anti-160 antibodies do not cross-react with U1A protein itself.

Purified recombinant U1A enhances CPSF directed polyadenylation and extends poly(A) tail length

The data above provide strong evidence that the U1A

protein and the 160-kD subunit of CPSF interact physically. Therefore, we wanted to establish whether this interaction correlates with a function. To this end, in vitro polyadenylation reactions were performed using a

Figure 5. Coimmunoprecipitation of U1A and 160-kD protein. (A) In vitro [35 S]Met-labeled transcription/translation products of U1A and 160-kD protein were prepared and are indicated by the input lanes, which represent 10% of the input used in the of the binding reactions (lanes 6,7). The 160-kD protein contained the g10 epitope tag. Lanes labeled g10 indicate the 160-kD protein and U1A protein immunoprecipitated or coimmunoprecipitated using an antibody to the g10 epitope; lanes Pre indicate similar immunoprecipitations using a control preimmune serum. Protein products present in each reaction are shown across the top of the gel. (B,C) Western blots of immunoprecipitations of cell extracts. HeLa cell nuclear extracts were immunoprecipitated using a control preimmune rabbit serum (Pre), with rabbit polyclonal antibodies against U1A (α U1A), or the 160-kD component of CPSF (α 160). The precipitates were separated on a 12.5% SDS-polyacrylamide gel (B) or a 10% gel (C). The immunoprecipitated proteins were treated with Laemmli sample buffer prior to boiling (B) or in Laemmli buffer without β -mercaptoethanol (C). After transfer to nitrocellulose, the blots were probed with the anti-U1A mouse monoclonal antibody 1E1 at a dilution of 1:500. The bands representing the U1A protein are indicated at right. After antibody binding, the blot in C was washed more extensively than the blot in B.

precleaved SV40 late RNA substrate (Fig. 6A, lane 1), which can be polyadenylated in vitro by the addition of purified CPSF and PAP (see Materials and methods; Fig. 6A, lane 2). To test the effect of U1A protein on polyadenylation, increasing amounts of purified, bacterially expressed, His-tagged U1A protein (Fig. 2B) were added to the reaction. The addition of the purified U1A (Fig. 6A, lanes 3–5; ~1.2, 3.8, and 7.5 pmoles, respectively) caused a concentration-dependent increase in total poly-

Figure 6. In vitro polyadenylation is enhanced by the addition of recombinant U1A. (A) In vitro polyadenylation reactions were performed using 32 P-labeled precleaved SV40 substrate RNA (lane 1), purified CPSF, PAP, and ATP (see Materials and methods). Lane 2 represents such a polyadenylation reaction. Purified His-tagged U1A (see Fig. 2B) was added to the reactions in increasing concentrations (lanes 3–7; 1.3, 3.8, 7.5, 17, and 20 pmoles, respectively). In lanes 8–13 similar polyadenylation reactions were treated with the same increasing amounts of heat-denatured (boiled for 5 min) His-tagged U1A. In lanes 14–19 similar polyadenylation reactions were treated with the same increasing amounts of purified His-tagged TFIIB. (B) An experiment similar to A where additional concentrations between 7.5 and 20 pmoles of U1A protein were tested. In addition, size markers (M) were included to quantitate the increase in poly(A) tail length (see Table 1). (Lane 1) The migration of the unreacted 32 P-labeled precleaved SV40 substrate RNA (Pre); (lane 2) the polyadenylation reaction with purified CPSF, PAP, ATP, and no added U1A; (lanes 3–8) the effect of added His-tagged U1A at amounts of 1.3, 3.8, 7.5, 10, 15, 20 pmoles, respectively.

adenylation. Quantitation of these data indicated that at 7.5 pmoles of U1A protein at least three times more substrate RNA was polyadenylated compared to the reaction with no added U1A. In addition, the increased utilization of precursor was accompanied by a concurrent increase in the length of the poly(A) tail. The effective range of U1A concentrations was ~15- to 100-fold greater than the concentrations of RNA and CPSF (which were approximately equimolar). This apparent requirement of a molar excess of His-tagged U1A may indicate that a fraction of the recombinant protein was inactivated during purification and/or reflects the relatively low affinity nature of the in vitro interaction. In contrast to this activation, U1A levels of ~15 pmoles and higher resulted in a sharp and dramatic inhibition of polyadenylation (Fig. 6A, lanes 6,7). The reason for this inhibition by these higher levels of U1A is unknown; however, it may reflect inhibition of PAP as has been suggested for the autoregulation of polyadenylation of U1A's own mRNA (Gunderson et al. 1994; see Discussion). In Figure 6A, lanes 8–13 show that the equivalent amounts of heat-denatured U1A protein neither activated nor inhibited polyadenylation. Likewise, lanes 14–19 show that the addition of equivalent amounts of a similarly purified, heterologous protein, His-tagged *Drosophila* TFIIB, or TATA-binding protein (TBP) (data not shown), had no effect on the polyadenylation reactions. These data suggest that the effects of U1A protein on CPSF-directed polyadenylation were specific.

The effect of U1A protein in a nonspecific polyadenylation reaction (Ryner et al. 1989a) was tested using the same substrate RNA, purified PAP, and Mn^{2+} (data not shown). Under these conditions U1A produced no positive effects on polyadenylation over the same concentration range, suggesting that stimulation of polyadenylation by U1A results from an interaction with CPSF. However, inhibition was again detected at high U1A concentrations, supporting the view that this reflects an interaction with PAP.

Figure 6B shows a similar experiment where more points were tested between 7.5 and 20 pmoles of U1A to define better the point of transition between activation and inhibition by U1A. In addition, size markers were included to quantitate the increase in poly(A) tail length. The data shows that the maximum positive effect on polyadenylation occurred with ~7.5 pmoles of U1A (lane 5) and that at 10 pmoles of U1A (lane 6) inhibition had begun and was complete at 20 pmoles (lane 8).

Measurements of poly(A) tail lengths were taken for the reactions that contained (1) no added U1A (lane 2), (2) 3.8 pmoles of U1A (lane 4), and (3) 7.5 pmoles of U1A (lane 5), which corresponds to the maximum effect in these experiments. The minimal and maximal poly(A) tail lengths were determined as well as the poly(A) tail length of the bulk of the RNA measured at the middle of the darkest region of the polyadenylated RNA. The data (Table 1) show that at 3.8 pmoles of U1A the tail length of the bulk of the RNA was increased by 20 residues, and at 7.5 pmoles of U1A the length was increased by 70 residues. The maximum poly(A) tail length was in-

Table 1. The effect of U1A on poly(A) tail length

Amount of U1A (pmole)	Length		
	maximum	bulk	minimum
0	150	80	25
3.8	210	100	30
7.5	260	150	30

creased by 60 using 3.8 pmole of U1A and by 110 with 7.5 pmole of U1A.

U1A protein enhances the binding of CPSF to AAUAAA-containing substrates

Electrophoretic mobility shift assays (EMSAs) were next performed to determine whether the interaction between U1A protein and CPSF affected binding of CPSF to AAUAAA-containing RNA. Figure 7 shows the mobility shift mediated by 30 and 60 ng of CPSF alone (lanes 7 and 11, respectively; see Material and methods). Consistent with previous data (Bienroth et al. 1991; Gilmartin and Nevins 1991; Murthy and Manley 1992), the CPSF–RNA interaction was relatively weak by itself. However, the addition of 1.3, 3.8, and 7.5 pmole of His-tagged U1A (lanes 4–6 for 30 ng of CPSF and lanes 8–10 for 60 ng of CPSF) enhanced CPSF binding in a concentration-dependent manner. Although the addition of higher amounts of U1A to the polyadenylation reaction inhibited polyadenylation, as described in Figure 6, these higher amounts of U1A did not decrease CPSF binding (not shown), further supporting the view that inhibition of polyadenylation results from interactions with PAP.

The addition of 1.2, 3.8, and 7.5 pmole of His-tagged U1A to the RNA without CPSF (lanes 1–3) indicated an interaction of U1A with the RNA, but these complexes migrated considerably faster compared with the mobility shift caused by CPSF. This may represent binding of U1A to the upstream sites of the SV40 RNA (Lutz and Alwine 1994). In any case, the effect of adding U1A plus CPSF was much greater than the additive binding of U1A and CPSF separately. Thus, our data indicate that U1A can mediate a significant enhancement or stabilization of the binding of CPSF to the AAUAAA-containing substrate RNA.

Discussion

In nature polyadenylation is frequently coordinated with splicing. Such coordination is suggested by the exon definition model proposed by Berget and co-workers (Roberson et al. 1990; Berget 1995). This model suggests an interaction between components of the splicing apparatus and the polyadenylation complex to define the last exon of an mRNA. Such interactions have been suggested by experiments using a coupled *in vitro* splicing and polyadenylation system (Niwa et al. 1990; Niwa and Berget 1991). These data showed that mutations in the

polyadenylation signal, which inhibited polyadenylation, also caused a decrease in the efficiency of splicing, that is, removal of the last intron. Likewise, mutations in the 3'-splice site of the last exon, which inhibited splicing, also caused inhibition of polyadenylation. Analogous transfection experiments have provided similar results *in vivo* (Chiou et al. 1991; Nesic et al. 1993; Nesic and Maquat 1994).

The role of snRNPs or other splicing factors in polyadenylation has been disputed in the past. Despite earlier suggestions to the contrary (for review, see Manley 1988), the purified, basal polyadenylation apparatus does not contain an RNA or snRNP component (Takagaki et al. 1989; Bienroth et al. 1991; Murthy and Manley 1992). However, several lines of evidence have suggested a role, or roles, for the U1 snRNP in nuclear polyadenylation. First, several studies showed that anti-snRNP antibodies (anti-Sm and anti-U1 snRNP) could inhibit *in vitro* polyadenylation reactions (Moore and Sharp 1984; Hashimoto and Steitz 1986; Raju and Jacob 1988). Second, experiments using the SV40 late polyadenylation signal suggested that a direct interaction between U1 snRNP and a binding site in the precursor RNA could influence polyadenylation efficiency (Wassarman and Steitz 1993). Finally, we have previously shown that U1A protein interacts with the elements of the SV40 late polyadenylation signal (Lutz and Alwine 1994) and that events disrupting the interaction significantly inhibit *in vitro* polyadenylation.

The above data suggest that the U1A protein and U1 snRNP perform an important role in polyadenylation. The finding that the U1A protein may bind to elements close to the AAUAAA of some polyadenylation signals

Figure 7. EMSAs demonstrate enhanced binding of CPSF to AAUAAA-containing RNA in the presence of U1A. The mobility shift of SV40 substrate RNA (Pre) was determined using increasing amounts of His-tagged U1A protein either alone (lanes 1–3) or in the presence of 30 ng of CPSF (lanes 4–6) or 60 ng of CPSF (lanes 8–10). The mobility shifts caused by 30 and 60 ng of CPSF alone are shown in lanes 7 and 11, respectively. The amount of His-tagged U1A in lanes 1, 4, and 8 was 1.3 pmole; in lanes 2, 4, and 9 was 3.8 pmole, and in lanes 3, 6, and 10 was 7.5 pmole.

(e.g., SV40 late) led us to suggest that this interaction occurs to facilitate a protein-protein interaction between the U1 snRNP and the cleavage and polyadenylation complex associated with the AAUAAA. This putative protein-protein interaction could account for a linkage between splicing and polyadenylation. The studies reported here confirm that such an interaction can occur between the U1A protein and the 160-kD subunit of CPSF and that this interaction is functionally significant.

In our studies we noted that U1A protein increased polyadenylation mediated by CPSF and PAP at lower concentrations but had an inhibitory effect at higher concentrations. The occurrence of this inhibition may reflect an inhibitory effect of free U1A protein on PAP similar to that suggested for the autoregulation of polyadenylation of U1A's own mRNA (Boelens et al. 1993; van Gelder et al. 1993; Gunderson et al. 1994). This involves two molecules of free U1A protein binding through their RRM1 domains to two adjacent sites upstream of the AAUAAA on the pre-mRNA encoding U1A protein (van Gelder et al. 1993; Gunderson et al. 1994). This complex facilitates an interaction with PAP that inhibits polyadenylation of the U1A pre-mRNA. Interestingly, the protein-protein interaction with PAP appears to require the carboxy-terminal half of U1A, as a peptide consisting of the amino-terminal 110 amino acids was unable to bind PAP and did not inhibit U1A pre-mRNA polyadenylation, although it retained the ability to bind to the pre-mRNA (Gunderson et al. 1994). In contrast, our data suggest that the interaction between the 160-kD subunit of CPSF and U1A is mediated through the amino-terminal 134 amino acids of U1A. Taken together, these data imply that the positive and negative effects of free U1A on polyadenylation may result from concentration-dependent interactions of the amino- and carboxy-terminal regions of the protein with CPSF and poly(A) polymerase, respectively.

The difference between the positive and negative effects of U1A may also depend on the specific binding site that the protein recognizes on the RNA, that is, U1 RNA-like sites as in U1A mRNA or upstream elements like those found in the SV40 late polyadenylation signal. However, our preliminary results suggest that deletion of the SV40 upstream elements in precleaved polyadenylation substrates has at most a modest effect on the ability of U1A to enhance CPSF-dependent polyadenylation (K.G.K. Murthy and J.L. Manley, unpubl.). We believe that in the purified, reconstituted system the positive effect of U1A is driven primarily by protein-protein interactions, perhaps facilitated by specific or nonspecific contacts between U1A and the substrate RNA. However, *in vivo* or in crude nuclear extracts (Lutz and Alwine 1994), the interactions with upstream elements may be needed for U1A to exert its full influence on CPSF.

The activation of polyadenylation by U1A that we observed correlates with enhanced binding of CPSF to the AAUAAA-containing SV40 RNA substrate. Consistent with this, similar analyses have previously suggested slight stabilization by U1A of CPSF binding to the U1A

RNA polyadenylation site (Gunderson et al. 1994), although these studies did not establish this point conclusively nor did they examine the possible functional significance. From the overall results we suggest that at appropriate levels U1A interacts with CPSF, helps stabilize its interactions with the polyadenylation signal, and thus increases polyadenylation efficiency. These data provide support for the existence of communication between a splicing component and the polyadenylation complex. However, the results of the present *in vitro* studies, which used purified, recombinant U1A protein, indicate that U1A alone can function as a significant affecter. It is interesting to consider that this may provide a role for free U1A in nuclear polyadenylation. On the other hand, the *in vitro* results may mimic a function of U1A that it naturally performs as part of the U1 snRNP. If the effects are mediated via U1 snRNP *in vivo*, then it is necessary to consider the spliceosome and all of the other splicing factors that could, potentially, mediate communication between splicing and polyadenylation. This provides intriguing possibilities for intricate and precise gene expression control through modulation of last exon definition and nuclear polyadenylation.

Materials and methods

Plasmids

pETg10:A has been described previously (Lutz-Freyermuth et al. 1990) and was used as template for *in vitro* transcription and translation of U1A protein with the g10 epitope tag. pGEM2:A was prepared by removing the *Bam*HI-*Eco*RI fragment from pETg10:A and placing it into pGEM2 (Promega). This construct was used to prepare U1A protein without the epitope tag by *in vitro* transcription and translation. A vector for the production of His-tagged U1A was prepared by placing this same fragment in pRSET (Invitrogen). pGEX2T-FLA (Bentley and Keene 1991) was a generous gift of J. Keene and contains the full-length U1A cDNA subcloned into the *Bam*HI site of pGEX2T. GST fusions of the amino- and carboxy-terminal halves of U1A protein (pGEX3X-Aamino and pGEX3X-Acarb) were generated by PCR of U1A cDNA regions corresponding to amino acids 1–134 and 135–282, respectively, using complementary primers. The primers also contained *Bam*HI (5') and *Eco*RI (3') recognition sites to allow in-frame insertion. After digestion with these enzymes, the fragments were ligated into *Bam*HI and *Eco*RI-digested pGEX3X. The constructs were transformed into *E. coli* HB101. Recombinants were both sequenced and assayed for expression of appropriately sized fusion protein. pET3a-*Bam*HI is a vector that can be used for the *in vitro* transcription/translation of the 160-kD protein. It was prepared by cloning the 160-kD protein cDNA into the *Bam*HI site of pET3a (Murthy and Manley 1995). This vector provides the g10 tag on the protein.

Antisera

Anti-T7 g10 epitope tag (anti-g10 tag) antibody was purchased from Novagen (Madison, WI). Anti-Sm monoclonal antibody (Y12) was a gift of Dr. Joan Steitz (Yale University, New Haven, CT). Anti-U1A rabbit polyclonal antibodies were described previously (Lutz and Alwine 1994). Monoclonal antibody 1E1 was prepared against U1A protein in mice. Polyclonal anti-160-kD

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antibodies were prepared in rabbits using a 110-kD fragment of recombinant 160-kD protein (Murthy and Manley 1995).

In vitro transcription and translation

Coupled in vitro transcription and translation reactions were performed using the Promega TNT system, using procedures provided by the manufacturer.

Coimmunoprecipitation assays

Approximately equimolar amounts of in vitro-transcribed/translated proteins were mixed and incubated at 30°C for 15 min in KNET (50 mM Tris-HCl at pH 7.4, 20 mM KCl, 80 mM NaCl, 2 mM EGTA, 0.05% NP-40). After transfer to ice the mixture was immunoprecipitated using anti-g10 tag monoclonal antibody (Novagen), and the immunoprecipitated proteins were collected and analyzed by SDS-PAGE as described previously (Lutz and Alwine 1994). In an alternate approach, in vitro-transcribed/translated proteins ($\sim 1.5 \times 10^5$ cpm of each) were co-incubated at 37°C for 30 min and immunoprecipitated with 1 μ l of anti-g10 tag monoclonal antibody (Novagen) essentially as described by Kohtz et al. (1994). Immunoprecipitated proteins were separated on a 9% SDS-polyacrylamide gel and then detected by fluorography.

Western blots

Samples were separated by electrophoresis on 12.5% SDS-polyacrylamide gels. For the analysis of fractions from the CPSF purification samples 30- μ l samples were used. The gels were then electroblotted to ECL Hybond (Amersham) or BA85 nitrocellulose (S&S) at 200 mA overnight at 4°C in buffer containing 192 mM glycine, 25 mM TRIS, and 20% methanol. Blots were blocked in Blotto (5% nonfat dry milk, 1 $\times$ PBS, 0.1% NP-40) for 3 hr at room temperature and then incubated at room temperature with the primary antibody diluted in Blotto for 1–2 hr. Primary antibodies included rabbit anti-U1A antibodies (diluted 1:500), rabbit anti-160-kD antibodies (diluted 1:100), mouse anti-Sm monoclonal antibody (diluted 1:1000), or anti-U1A monoclonal antibody 1E1 (diluted 1:800). After washing three to five times with 1 $\times$ PBS plus 0.1% NP-40, the blots were incubated for 2 hr at room temperature with the secondary antibody (either peroxidase-conjugated goat anti-rabbit or peroxidase-conjugated goat-anti mouse), which was diluted 1/8000 in 1% BSA, 1 $\times$ PBS, and 0.1% NP-40. The blots were washed again in 1 $\times$ PBS, and luminescence was developed using the ECL kit (Amersham). Blots were stripped prior to reprobing as directed by the kit's manufacturer.

Immunoprecipitation followed by Western blotting

Pansorbin (Calbiochem) beads were washed three times with NET-1 (150 mM NaCl, 5 mM EDTA, 50 mM Tris at pH 7.4, 0.5% NP-40). Washed beads (100 μ l) were added to either 100 μ l of purified anti-160 rabbit antiserum (purified and concentrated by DEAE chromatography and $(\text{NH}_4)_2\text{SO}_4$ precipitation; 10 μ g/ μ l), 25 μ l of anti-U1A rabbit antiserum (60 μ g/ μ l) plus 75 μ l of PBS, or 25 μ l of rabbit prebleed antiserum (57 μ g/ μ l) plus 75 μ l of PBS. The antibodies were allowed to bind to the beads on a Nutator (Adams) at 4°C for 7 hr. The beads were then washed three times with NET-1 and resuspended in 300 μ l of buffer (20 mM HEPES at pH 7.6, 20% glycerol, 100 mM KCl, 0.2 mM EDTA) and were allowed to bind to 100–150 μ l (3.4 μ g/ μ l) HeLa nuclear extract (Schek et al. 1992) on a Nutator at 4°C overnight (~ 12 hr). The beads were then washed four times with NET-1,

applied to SDS-polyacrylamide gels, transferred to nitrocellulose, and probed as described under Western blotting.

Far Western protein blotting

The procedure of Lee et al. (1991) was followed with minor modifications. His-U1A (1.8 μ g) was separated on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose as described above. The protein blot was denatured and subsequently renatured by washing as follows in 1 $\times$ CZ buffer [20 mM HEPES at pH 7.9, 17% glycerol, 0.1 M KCl, 5 mM MgCl_2 , 0.1 mM ZnCl_2 , 0.1 mM EDTA, 2 mM dithiothreitol (DTT)] plus 0.5 mg/ml of BSA, 0.02% polyvinylpyrrolidone (PVP), and 6 M guanidine-HCl for 30 min, one time; 1 $\times$ CZ plus 0.5 mg/ml of BSA, 0.02% PVP, and 0.1 M guanidine-HCl for 1 hr, twice; 1 $\times$ CZ plus 0.5 mg/ml of BSA and 0.02% PVP for 2 hr, twice; and 10 mM HEPES (pH 7.9) plus 5% BSA for 1 hr. The blot was then incubated with $\sim 2 \times 10^6$ cpm of [^{35}S]Met-labeled protein (160 kD or IEP72) prepared by the TNT system. To reduce background the labeled proteins were first mixed with an equal volume of 3 mM cold methionine for 10 min on ice prior to addition to the blot. The blot was probed overnight with labeled protein as described above in 2 ml of 0.5 $\times$ CZ per strip and washed with 50 ml of 0.5 $\times$ CZ for 30 min, twice. All washings and incubations with protein probes were performed at room temperature.

GST fusion protein-binding assays

GST-A fusion proteins were expressed and purified under conditions essentially as described by Gruda et al (1993). Fusion proteins were checked on silver- or Coomassie-stained gels to ascertain purity and concentration. Equivalent amounts were immobilized on glutathione-agarose beads and incubated with 2.5×10^5 cpm of each in vitro-transcribed/translated protein. For the RNase treatment of the fusion protein binding assays, the bound and washed samples were incubated with 22 μ g of RNase A and 1×10^4 units of RNase T1 for 30 min at room temperature with shaking. The reactions were then washed further prior to analysis on polyacrylamide gels.

Polyadenylation assays

In vitro polyadenylation experiments were performed using highly purified CPSF, PAP, and ^{32}P -labeled SV40 late substrate RNA (linearized at the *HpaI* site) as described (Murthy and Manley 1992). Briefly, the reactions contained 2 ng of RNA, 0.2 μ l (80 ng) of extensively purified (10%–20% pure) HeLa PAP (Mono S fraction; Ryner et al. 1989a), 2 μ l (~ 20 ng) of homogeneous calf thymus CPSF (glycerol gradient fraction; Murthy and Manley 1992), 8 mM HEPES (pH 7.9), 40 mM NaCl, 0.08 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 2.5% PVA, 0.4 μ g of *E. coli* tRNA, and 1 mM ATP in a final reaction volume of 12.5 μ l. The reactions were incubated at 30°C for 45 min. His-U1A protein, or other control proteins (*Drosophila* TFIIB or TBP; the kind gift of Chi Li, Columbia University, New York, NY) were added to these reactions at various concentrations as detailed in the text and figure legends.

Bacterial expression of proteins

His-tagged U1A was expressed from pRSET-HisA in *E. coli* BL21(DE3) cultures. The tagged protein was purified on nickel-agarose (His-Bind resin) according to procedures provided by the manufacturer (Novagen). The protein was then dialyzed into buffer containing 150 mM NaCl, 10 mM HEPES at pH 7.6 and was analyzed for purity on a Coomassie-stained polyacrylamide

gel. The concentration was ~1.2 mg/ml. His-tagged TFIIB was prepared in a similar fashion from pRSET and had a concentration of ~1.2 mg/ml.

EMSAs

Analyses by EMSA were performed as described by Murthy and Manley (1992). Briefly, the reactions were prepared as described above for the polyadenylation reactions, except that the CPSF fraction was from the poly(U) purification step of Murthy and Manley (1992), which was further purified by Superose 6 chromatography (~30% pure), and incubation was at 30°C for 10 min. Then 6.25 μ l of each sample was loaded directly onto 4% nondenaturing gels (80:1 acrylamide/bisacrylamide). Electrophoresis was carried out in Tris-glycine buffer (25 mM Tris base, 25 mM boric acid, 1 mM EDTA) at room temperature at 20 mA.

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