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The snRNP-free U1A (SF-A) complex(es): Identification of the largest subunit as PSF, the polypyrimidine-tract binding protein-associated splicing factor

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ABSTRACT

We have previously shown that a specific monoclonal antibody prepared against the U1A protein, MAb 12E12, is unique in its ability to recognize a form of U1A which is not associated with the U1snRNP. This unique form of U1A, termed snRNP-free U1A or SF-A, was found to be complexed with a novel set of non-snRNP proteins (O'Connor et al., 1997, *RNA* 3:1444-1455). Here we demonstrate that the largest protein in these SF-A complex(es), p105, is the polypyrimidine-tract binding protein-associated factor (PSF), an auxiliary splicing factor. We show that PSF copurifies and co-immunoprecipitates with SF-A from 293T cell nucleoplasm and that it interacts with SF-A in vitro. In addition, we show that MAb 12E12 inhibits both splicing and polyadenylation in an in vitro coupled splicing and polyadenylation reaction. This suggests that SF-A and/or the SF-A complex(es) perform an important function in both processing reactions and possibly in last exon definition.

Keywords: polyadenylation; RNA processing; SF-A complex; snRNP-free U1A (SF-A); splicing; U1snRNP

INTRODUCTION

Formation of mature mammalian mRNA requires several precise RNA processing events, including splicing and polyadenylation. Many cellular factors are involved in RNA processing. The best characterized of these are the small nuclear ribonucleoprotein particles (snRNPs) that, along with other non-snRNP factors, form the spliceosome necessary for mRNA splicing (reviewed in Moore et al., 1993; Staley & Guthrie, 1998). Polyadenylation is the process by which the 3' end of the mature mRNA is formed through specific endonucleolytic cleavage of the precursor RNA and the addition of approximately 250–300 unencoded adenosine residues. Polyadenylation is also a complex process, in-

volving many protein factors and RNA signals (reviewed in Wahle & Keller, 1996; Proudfoot, 1996; Colgan & Manley, 1997). Although snRNPs are not immediately thought of as polyadenylation factors, experimental evidence from several labs has suggested that splicing and polyadenylation may be functionally linked (Niwa et al., 1990; Niwa & Berget, 1991; Chiou et al., 1991; Nesic et al., 1993; Nesic & Maquat, 1994). These data support the exon definition model for splicing of mammalian mRNA proposed by Berget and colleagues (see Berget, 1995, for review). This model suggests that components of both splicing and polyadenylation complexes interact to define the last exon and affect the efficiencies of both polyadenylation and last intron removal.

In agreement with the exon definition model and the possible involvement of snRNPs in last exon definition, we have shown that components of the U1 snRNP function in polyadenylation. We have previously demonstrated that the U1snRNP-A protein (U1A) can bind to the SV40 late polyadenylation signal (Lutz & Alwine,

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1994), and that purified U1A can interact with a component of the polyadenylation complex, the 160 kDa subunit of cleavage-polyadenylation specificity factor (CPSF) (Lutz et al., 1996). Using purified components (CPSF, poly(A) polymerase, and precleaved RNA) we have shown that the addition of purified bacterially expressed U1A caused a concentration-dependent increase in both the overall level of polyadenylation and in poly(A) tail length (Lutz et al., 1996). In agreement with this result we have shown that the purified recombinant U1A stabilized the interaction of CPSF with the AAUAAA-containing substrate RNA. It is important to emphasize that in both the *in vitro* polyadenylation and binding analyses the U1A protein functioned in a form which was free of other U1snRNP components. We refer to this form as snRNP-free U1A, or SF-A.

Our previous *in vitro* data suggests that specific concentrations of SF-A can mediate a general enhancement of polyadenylation (Lutz et al., 1996; O'Connor et al., 1997). Other experiments have indicated that U1A can also mediate a very specific negative feedback regulatory mechanism affecting the polyadenylation of its own mRNA. This involves specific interactions with both the pre-mRNA encoding U1A and poly(A) polymerase (Gunderson et al., 1994, 1997). These findings raise the question of how much U1A protein is present as SF-A in the cell. It has been assumed that most, if not all, of the cellular U1A protein is tightly bound to U1 RNA as part of the snRNP. This assumption was based upon the high affinity ($K_d \sim 10^{-9}$) of U1A for U1 RNA (Lutz-Freyermuth et al., 1990; Jessen et al., 1991), and upon the negative autoregulation of polyadenylation of U1A's own message (Boelens et al., 1993).

Recently we demonstrated that at least two classes of U1A can be detected in human cell nucleoplasm: U1A that is associated with the U1snRNP, and SF-A that is complexed with a distinct set of non-snRNP proteins, that is, the SF-A complex(es) (O'Connor et al., 1997). Based upon the quantitation of cellular levels of U1A (Baserga & Steitz, 1993) we estimated that approximately 3% of the total U1A protein in the cell is part of the SF-A complex. This corresponds to approximately 30,000 molecules. Our previous data also indicated that SF-A has a physiologically relevant function: specifically, an antibody which is specific for SF-A and not snRNP-bound U1A can inhibit *in vitro* polyadenylation of an SV40 late polyadenylation substrate RNA.

As mentioned above SF-A has been shown to be in complex(es) with other non-snRNP proteins (O'Connor et al., 1997). In this article, we demonstrate (1) that the largest protein in the SF-A complex(es), p105, is PSF, the polypyrimidine-tract binding protein-associated splicing factor (Patton et al., 1993); (2) that PSF interacts with U1A both *in vitro* and *in vivo*; and (3) that SF-A has effects on both splicing and polyadenylation in a coupled splicing and polyadenylation reaction.

RESULTS

Peptide sequencing of the p105 protein from the SF-A complex reveals that it is the splicing factor PSF

In our previous work documenting the existence of SF-A we noted that SF-A migrated in sucrose gradients as a complex(es) containing a number of other non-snRNP proteins (p58, p59, p63, p65, and p105; O'Connor et al., 1997). To define the proteins in the complex we have prepared partially purified complex(es) by large-scale immunoprecipitation. Purification and sequence analysis was most successful with the largest protein, p105, which migrated in preparative gels in a position that was relatively free of other proteins. Figure 1 shows an Amido Black-stained PVDF membrane to which partially purified complex(es) has been transferred from a polyacrylamide gel. Because of the partial purification of the complex(es), bands migrating larger than p105 are apparent. As previously shown, these proteins are removed with further purification (O'Connor et al., 1997). The indicated p105 band was excised and peptide sequenced by the Cold Spring Harbor Peptide Sequencing Facility. The pro-

FIGURE 1. Partial purification of SF-A complex(es). Shown is a preparative SDS-PAGE separation of partially purified SF-A complex(es) (SF-A comp) transferred to PVDF membrane. The proteins were visualized by Amido Black staining (see Materials and Methods). The molecular weight markers are shown in kiloDaltons. The position of p105 and U1A protein are indicated. The bracket marked with the asterisk indicates the positions of other proteins (58–65 kDa) which co-immunoprecipitate with SF-A and appear to be part of the complex(es) (O'Connor et al., 1997). The p105 band was excised for peptide sequencing.

Sequence of PSF

MSRDRFRSRGGGGGGFHRGGGGGRGGLHDFRSPPPGMGLNQNRGPMGPGPGQ
 SGPKPIPPPPPHQQQPPPPQPPPPQPPPHQPPPHQPPHQQQPPPPPDSSKPV
 VAQGGPGAPGVGSAPPASSSAPPATPPTSGAPPGSGPGPTPTPPPAVTSAPPGAPP
 TPPSSGVPTTPPQAGGPPPPAAVPGPGPGPKQGGPGGPKGGKMPGGPKPGGGP
 GLSTPGGHPKPPHRGGGEPRGGRQHHPYHQHHQGPPPGGPGGRSEEKISDSEG
 FKANLSLLRRPGEKTYTQRCRLFVGNLPADITEDEFKRLFAKYGEPGEVFINKGKGFGF
 IKLESRALAEIAKAELDDTPMRGRQLRVRFATHAAALSVRNLSPYVSNELLEAFSQFG
 PIERAVVIVDDRGRSTGK**GIVEFASK**PAARK**AFERCSEGVFLLTTTPRPVIVE**PL
 EQLDDEGLPEKLAQKNPMYQKERETPPRFAQHGTFEYEYSQRWKSLEDEMEKQQR
 QVEKNMKDAKDKLESEMEDAYHEHQANLLRQDLMRQEELRRMEELHNQEMQKRKE
 MQLRQEEERRRREEEMIRQREMEEQMRRQREESYSRMGYMDPRERDMRMGGGG
 AMNMGDPYGS GGQKFPPLGGGGGIGYEANPGVPPATMSGSMGSDMRTERFGQG
 GAGPVGGQGPRGMGPGTPAGYGRGREEYEGPNKKPRF

Peptides Sequenced

(A)FERXSEGVFLLXTTXXX(V)I(V)E peptide #1
 GIVEFAS(K) peptide #2

FIGURE 2. Peptide sequencing reveals the identity of p105 as the splicing factor PSF. The amino acid sequence of PSF is shown at the top. The two peptides sequenced from p105 are shown below. Both peptides are shown in bold type within the sequence of PSF.

tein was digested with *Achromobacter* protease I, and the resulting peptides were HPLC purified. Two peptides were chosen for sequencing on an ABI Procise 494 automated peptide sequencer. The resulting sequences were used to search the GenBank databases to identify the protein. This analysis revealed that both peptide sequences were found in PSF (Patton et al., 1993). Figure 2 shows the sequences of the two peptides and where they are located within the sequence of PSF.

Sucrose gradient cofractionation of PSF and U1A

We next wanted to confirm that PSF was a component of the SF-A complex(es). Previously we have shown that the SF-A complex(es) in human 293T cell nucleoplasmic extracts migrates in fractions 7–11 of a 5–30% sucrose gradient. In contrast, the U1snRNP is found in fractions 17–23 (O'Connor et al., 1997). We prepared similar sucrose gradients using human 293T cell nucleoplasm, separated the resulting fractions by SDS-PAGE, and transferred the gel to nitrocellulose. The resulting Western blot was probed with a rabbit polyclonal anti-PSF antibody obtained from J. Patton (Patton et al., 1993). Figure 3 shows that the p105 band migrating in fractions 9–11 is specifically detected by the anti-PSF antibody. In addition, no other PSF is detected in the gradient; the presence of a PSF signal in fraction 25 is the result of "spill over" from the HeLa lane next to it. To ascertain the position of U1A in the gradient, the blot was then probed (without stripping) with a rabbit polyclonal antibody that recognizes U1A (Lutz & Alwine, 1994). U1A was found in fractions 9–11, where the SF-A complex(es) migrate, as well as in

fractions 17–23, where the U1snRNP migrates. These data confirm that PSF migrates in the portion of the gradient where the SF-A complex(es) are found and suggests that PSF is part of the SF-A complex(es).

FIGURE 3. Western blot of sucrose-gradient fractions shows that PSF and U1A comigrate in the SF-A-containing fractions. Sucrose gradients of 5–30% were prepared using human 293T cell nucleoplasm. One-milliliter fractions were taken and 30 μ L of every other fraction were separated by SDS-PAGE. Fraction 1 represents the top of the gradient. The gel was transferred to nitrocellulose for Western analysis. The transfer was probed first with a rabbit polyclonal antibody specific for PSF and visualized using horseradish peroxidase conjugated anti-rabbit secondary antibody and ECL reagents (Amersham). The blot was then probed, without stripping, with a rabbit polyclonal antibody specific for U1A and visualized in the same manner. The positions of PSF and U1A are noted on the right; the relative migration positions of the SF-A complex(es) and the U1snRNP are noted below. PSF detected in lane 25 is because of lane contamination from the adjacent HeLa input lane.

Analysis of the interaction between U1A and PSF by co-immunoprecipitation

Monoclonal antibody 12E12 is an antibody that specifically recognizes the SF-A form of the U1A protein and precipitates the SF-A complex(es). This monoclonal antibody does not recognize U1A as part of the U1snRNP, presumably since its epitope is masked when U1A is bound to U1 RNA (O'Connor et al., 1997). To further confirm the presence of PSF in the SF-A complex(es), 293T cell nucleoplasm was immunoprecipitated with monoclonal antibody 12E12 or a negative control antibody (SP2/0). The resulting immunoprecipitates were analyzed by SDS-PAGE and transferred to nitrocellulose. The resulting Western transfer was probed with the polyclonal antibody specific for PSF (Fig. 4). PSF is found in the 12E12 immunoprecipitated sample but not in the SP2/0 precipitated sample, suggesting that PSF co-immunoprecipitates as part of the SF-A complex(es).

Examination of binding between GST-U1A and PSF

To further establish an interaction between PSF and U1A we analyzed in vitro protein-protein interactions between in vitro transcribed and translated ^{35}S -Met PSF and GST fusions with U1A. Fusion proteins used were: the glutathione binding moiety alone (GST); the full-length U1A protein fused to GST (GST-U1A); the amino-terminal half of U1A fused to GST (AA); and the carboxy-terminal half of U1A (AC). The GST moiety alone and each fusion protein were expressed in

FIGURE 4. Immunoprecipitated SF-A complex(es) contains PSF. Human 293T cell nucleoplasm was immunoprecipitated with either the SF-A specific monoclonal antibody 12E12 or a negative control SP2/0. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose for Western analysis. The transfer was probed with a polyclonal antibody specific for PSF. Molecular weight standards are indicated on the left; the positions of PSF is indicated on the right.

Escherichia coli and were purified on glutathione agarose (Lutz et al., 1996). The complexes of beads containing the GST-U1A fusion proteins and ^{35}S -PSF were washed extensively in increasing salt concentrations and the remaining bound proteins were analyzed by SDS-PAGE. Figure 5 shows PSF had no significant affinity for the GST moiety but bound specifically to GST-U1A. This interaction was unstable in wash buffer containing 250 mM NaCl, suggesting a significant ionic character to the interaction. Figure 5 also shows that PSF binds to the amino-terminal half of U1A, which contains RRM1 (the U1 RNA binding domain; Lutz-Freyermuth et al., 1990), but not to the carboxy-terminal half (which contains RRM2). These in vitro data confirm a specific protein-protein interaction between PSF and U1A.

Splicing and polyadenylation inhibition experiments

We have previously shown that addition of the purified SF-A specific monoclonal antibody, 12E12, to in vitro polyadenylation reactions inhibits polyadenylation using the SV40 late polyadenylation signal as the substrate (O'Connor et al., 1997). These data suggested that SF-A or the SF-A complex(es) have direct effects on the polyadenylation reaction. Given that we identified PSF, a known splicing factor, as one of the components of the SF-A complex(es), we asked whether monoclonal antibody 12E12 would affect both polyadenylation and splicing in a coupled in vitro splicing/polyadenylation reaction using a HeLa cell nuclear extract. The coupled splicing and polyadenylation substrate was MXSVL, a substrate previously used to study last-exon definition and the coupling of splicing and polyadenylation (Niwa et al., 1990; Niwa &

FIGURE 5. GST-U1A binds to in vitro transcribed and translated PSF. Shown are binding assays between in vitro transcribed/translated ^{35}S Met-labeled PSF and (1) the GST moiety alone (GST); (2) a GST fusion with U1A (GST-U1A); (3) a GST fusion with the amino-terminal half (AA 1–134) of U1A (GST-AA); and (4) a GST fusion with the carboxy-terminal half (AA 135–282) of U1A (GST-AC). Bound proteins were washed in increasing NaCl concentrations (mM) as indicated across the top. Bound proteins were separated by SDS-PAGE and visualized by autoradiography. Protein standards indicating the relative molecular weight are shown on the left. The input lane represents 10% of the total input in the binding reactions.

Berget, 1991). A diagram of the MXSVL substrate is shown at the bottom of Fig. 6; it consists of a splicing cassette from the adenovirus major late region attached to the SV40 late polyadenylation signal. The *in vitro* reactions (top of Fig. 6) were done in the presence of cordycepin 5'-triphosphate which inhibits the addition of the poly(A) tail after cleavage at the site of polyadenylation. Hence discrete bands representing the cleavage products are detected. The data (Fig. 6) show the migration of the substrate MXSVL RNA (Input) and the products of the coupled reaction are indicated: the polyadenylated-only product (S-P+), the spliced-only product (S+P-), and the fully processed spliced and polyadenylated product (S+P+). Preincubation of the extracts with buffer (No MAB lanes) or purified monoclonal antibodies showed that 10 μ g of the control monoclonal (α 810, against the cytomegalovirus immediate early proteins) had no effect whereas an equivalent amount of 12E12 inhibited all processing. Monoclonal antibody 12E12 is very specific for SF-A; under the conditions used in these experiments 12E12 does not immunoprecipitate U1 snRNP bound U1A (data not shown). Hence the data

in Fig. 6 suggest that SF-A or the SF-A complex(es) are involved in both polyadenylation and splicing.

DISCUSSION

In this article, we have identified the largest of the proteins in the novel SF-A complex(es), the p105 protein, as the splicing factor PSF. We have also shown that PSF comigrates and co-immunoprecipitates with the SF-A complex(es) and that it interacts with U1A *in vitro* by protein-protein interactions. PSF is an essential pre-mRNA splicing factor originally isolated because of its copurification and association with the polypyrimidine-tract binding protein (PTB) (Patton et al., 1993). The physiological relevance of this interaction has yet to be determined. PSF is also identical to spliceosome-associated protein (SAP) 102 (Bennett et al., 1992; Gozani et al., 1994). It is unclear, however, in which catalytic step of splicing PSF participates. Step I of pre-mRNA splicing results in cleavage of the 5' splice site, yielding a "free" 5' exon and a lariat intermediate (see Moore et al., 1993; Staley and Guthrie, 1998, for reviews). Step II involves ligation of the 5' exon to the

FIGURE 6. SF-A specific monoclonal antibody 12E12 inhibits both splicing and polyadenylation in an *in vitro* coupled splicing and polyadenylation reaction. The MXSVL splicing and polyadenylation substrate is shown at the bottom and is described in Results. Its migration in the 5% polyacrylamide-7 M urea gel is shown in the Input lane. MXSVL was added to *in vitro* coupled splicing and polyadenylation reactions where the HeLa nuclear extracts had been preincubated with buffer (No MAB lane), 10 μ g purified control monoclonal (α 810, against HCMV IE proteins) or 10 μ g of purified SF-A specific monoclonal antibody 12E12. The reaction products were separated on a 5% polyacrylamide-7 M urea gel. The processing products are S-P+: polyadenylated and not spliced; S+P-: spliced and not polyadenylated; S+P+: spliced and polyadenylated.

3' exon with displacement of the intron as a lariat product. While data has suggested that PSF plays a role in catalytic step II of splicing (Patton et al., 1993; Gozani et al., 1994), other studies suggest that a PSF-depleted extract retains catalytic step II activity (Lindsey et al., 1995).

The previously reported association between PSF and PTB suggests that one of the other proteins in the SF-A complex(es) may be PTB. This is particularly appealing since PTB is a 57 kDa protein and other proteins in the SF-A complex(es) are 58, 59, 60, and 63 kDa. However, examination of Western transfers of SF-A complex(es) using anti-PTB antibodies has failed to detect PTB (data not shown) under conditions where the antibody detected PTB in crude extracts. Hence we do not believe that PTB is in SF-A complex(es).

We have previously shown (O'Connor et al., 1997) that MAb 12E12, an antibody that specifically interacts with SF-A, can specifically inhibit in vitro polyadenylation of an RNA substrate consisting of the SV40 late polyadenylation signal. These data suggest that SF-A and/or the SF-A complex(es) perform a significant function in polyadenylation. The presence of PSF, a known splicing factor, in the SF-A complex(es) further suggests that the SF-A complex(es) may be significant in splicing and provides a link between splicing and polyadenylation as suggested in the exon definition model (see Introduction). Thus we examined the effect of MAb 12E12 on splicing and polyadenylation in a coupled in vitro splicing and polyadenylation reaction using a substrate well characterized in the coupled system. The data show that the anti-SF-A antibody inhibited both splicing and polyadenylation, suggesting that SF-A or the SF-A complex(es) performs a significant role in each process and may be a linkage between splicing and polyadenylation for definition of the last exon.

MATERIALS AND METHODS

Purification of SF-A complex(es) and peptide sequencing

SF-A complex(es) was purified for peptide sequencing using a large scale immunoprecipitation protocol. One milliliter of monoclonal antibody 12E12 ascites fluid was chemically coupled to 2 mL of prewashed Gamma Bind-Plus Sepharose (Pharmacia, capacity = 17 mg/mL) using dimethylpimelimide as previously described (Harlow & Lane, 1988). Nucleoplasm was prepared as described (O'Connor et al., 1997) from ten 150-mm plates of human 293T cells, and was incubated with the coupled antibody for 2 h at 4°C. The bound antigens were eluted in 0.1 M glycine pH 3. Eluted fractions were analyzed by SDS-PAGE (12% acrylamide 68:1). The proteins were visualized by silver staining the gels. The fractions containing the most abundant amounts of SF-A complex(es) were pooled and preparatively fractionated by SDS-PAGE (12% acrylamide 68:1) and transferred electrophoretically to polyvinylidene difluoride (PVDF)

membrane. The p105 band was visualized by staining with Amido Black (0.1% Amido Black in 10% acetic acid), excised, and subjected to peptide sequencing.

p105 from the PVDF membrane was digested with *Achromobacter* protease I in 50 mM Tris-HCl (pH 9.0) containing 0.05% Tween 20 for 24 h at 30°C. The peptide fragments were extracted with 50% acetonitrile containing 0.064% TFA. The digest was separated by HPLC (Hewlett Packard 1090) using a Vydac C18 column (1.0 × 250 mm, 10 micron). The peptides were sequenced on an automated protein sequencer (ABI Procise 494).

Sucrose-gradient fractionation followed by Western blotting

Sucrose gradients of 5–30% (w/v) were prepared using human 293T cell nucleoplasm as described previously (O'Connor et al., 1997). Briefly, gradients were centrifuged in a Beckman SW28 rotor at 25,000 RPM for 42 h at 4°C. Thirty 1-mL fractions were taken using a BioComp model 150 gradient fractionator. Thirty microliters of every other fraction were separated by SDS-PAGE (12.5% acrylamide 29:1) and were electrophoretically transferred to nitrocellulose. The membrane was subsequently blocked in PBS containing 5% nonfat dry milk. Primary antibody incubations were performed for 1 h at room temperature. Rabbit polyclonal anti-U1A antibodies (Lutz & Alwine, 1994) were diluted 1:750 in PBS containing 5% nonfat dry milk; rabbit polyclonal anti-PSF antibodies were diluted 1:1,000. Bound antibodies were detected by chemiluminescence (ECL system, Amersham) using a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Cappel) diluted 1:8,000 in PBS.

Immunoprecipitation followed by Western blotting

Samples were immunoprecipitated using monoclonal antibodies chemically coupled to Gamma Bind Plus Sepharose as described above. Immunoprecipitated proteins were then separated by SDS-PAGE (12.5% acrylamide, 29:1) and were transferred to nitrocellulose for Western blots as described above.

GST fusion protein assays

GST full-length U1A and sub-fragment fusion proteins were expressed and purified as previously described (Gruda et al., 1993; Lutz et al., 1996). Fusion proteins were analyzed by SDS-PAGE followed by Coomassie staining to ascertain purity and concentration. Equivalent amounts of fusion proteins were immobilized on glutathione-agarose beads and incubated at 4°C with in vitro transcribed and translated ³⁵S methionine labeled PSF (2.5 × 10⁵ cpm) (TNT system, Promega). The PSF construct used for the coupled transcription/translation was pET-PSF, a gift of J. Patton, Vanderbilt University. The bound protein complexes were then washed in NETN+ (20 mM TRIS pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM tosyl-lysine chlo-

romethyl ketone), as previously described (Gruda et al., 1993). The bound protein was analyzed by SDS-PAGE (12.5% acrylamide, 29:1).

Nuclear extracts and in vitro processing reactions

HeLa-S3 cell nuclear extracts were prepared as described by Moore & Sharp (1985), using cells obtained as a cell pellet from the National Cell Culture Center. In vitro splicing and polyadenylation reactions (25 μ L final volume) contained 32% (v/v) nuclear extract, 250 mM ATP, 1 mM cordycepin 5'-triphosphate (Sigma), 20 mM Creatine Phosphate (Sigma), 26% polyvinyl alcohol, 1 mM $MgCl_2$, 40 units of recombinant RNase-inhibitor (Promega) and approximately 5 fmol of ^{32}P -labeled MXSVL substrate RNA. For the antibody preincubation experiments, the nuclear extract was pre-aliquoted for each reaction into tubes containing the polyvinyl alcohol and preincubated on ice for 15 min in the presence of either Dig-nam D buffer, 10 μ g CMV 810 mAb (Chemicon), or 10 μ g 12E12 MAb. Monoclonal antibodies had been previously purified. Reactions were initiated by adding a reaction cocktail containing the remaining reaction components and incubated at 30 ° C for 35 min. Reactions were stopped by the addition of 8 volumes of 20 mM Tris (pH 7.5), 400 mM NaCl and 0.01% sodium dodecyl sulfate. Reaction products were extracted once with phenol-chloroform-isoamyl alcohol, ethanol precipitated, and analyzed on 5% polyacrylamide-7 M urea gels.

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REFERENCES

- Baserga SJ, Steitz JA. 1993. The diverse world of small ribonucleoproteins. In: Gesteland RF, Atkins JF, eds., *The RNA world*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 359–382.
- Bennett M, Michaud S, Kingston J, Reed R. 1992. Protein components specifically associated with prespliceosome and spliceosome complexes. *Genes & Dev* 6:1986–2000.
- Berget SM. 1995. Exon recognition in vertebrate splicing. *J Biol Chem* 270:2411–2414.
- Boelens WC, Jansen EJR, van Venrooij WJ, Stripeke R, Mattaj IW, Gunderson SI. 1993. The human U1A snRNP-specific protein inhibits polyadenylation of its own pre-mRNA. *Cell* 72:881–892.
- Chiou HC, Dabrowski C, Alwine JC. 1991. Simian virus 40 late mRNA leader sequences involved in augmenting mRNA accumulation via multiple mechanisms, including increased polyadenylation efficiency. *J Virol* 65:6677–6685.
- Colgan DF, Manley JL. 1997. Mechanism and regulation of mRNA polyadenylation. *Genes & Dev* 11:2755–2766.
- Gozani O, Patton JG, Reed R. 1994. A novel set of spliceosome-associated proteins and the essential splicing factor PSF bind stably to pre-mRNA prior to catalytic step II of the splicing reaction. *EMBO J* 13:3356–3367.
- Gruda MC, Zabolotny JM, Xiao JH, Davidson I, Alwine JC. 1993. Transcriptional activation by SV40 T antigen: Interactions with multiple components of the transcriptional complex. *Mol Cell Biol* 13:961–969.
- Gunderson SI, Beyer K, Martin G, Keller W, Boelens WC, Mattaj IW. 1994. The human U1A snRNP protein regulates polyadenylation via a direct interaction with poly(A) polymerase. *Cell* 76:531–541.
- Gunderson SI, Vagner S, Polycarpou-Schwarz M, Mattaj IW. 1997. Involvement of the carboxyl terminus of vertebrate poly(A) polymerase in U1A autoregulation and in the coupling of splicing and polyadenylation. *Genes & Dev* 11:761–773.
- Harlow E, Lane D, eds. 1988. *Antibodies: A laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Jessen TH, Oubridge C, Teo CH, Pritchard C, Nagai K. 1991. Identification of molecular contacts between the U1A small nuclear ribonucleoprotein and U1 RNA. *EMBO J* 10:3447–3456.
- Lindsey LA, Crow AJ, Garcia-Blanco MA. 1995. A mammalian activity required for the second step of pre-messenger RNA splicing. *J Biol Chem* 270:13415–13421.
- Lutz CS, Alwine JC. 1994. Direct interaction of the U1snRNP-A protein with the upstream efficiency element of the SV40 late polyadenylation signal. *Genes & Dev* 8:576–586.
- Lutz CS, Murthy KGK, Schek N, O'Connor JP, Manley JL, Alwine JC. 1996. Interaction between the U1snRNP-A protein and the 160-kD subunit of cleavage-polyadenylation specificity factor increases polyadenylation efficiency in vitro. *Genes & Dev* 10:325–337.
- Lutz-Freyermuth C, Query CC, Keene JD. 1990. Quantitative determination that one of two potential RNA-binding domains of the A protein component of the U1 small nuclear ribonucleoprotein complex binds with high affinity to stem-loop II of U1 RNA. *Proc Natl Acad Sci USA* 87:6393–6397.
- Moore CL, Sharp PA. 1985. Accurate cleavage and polyadenylation of exogenous RNA substrate. *Cell* 41:845–855.
- Moore MJ, Query CC, Sharp PA. 1993. Splicing of precursors to messenger RNA by the spliceosome. In: Gesteland RF, Atkins JF, eds. *The RNA world*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 303–358.
- Nesic D, Cheng J, Maquat LE. 1993. Sequences within the last intron function in RNA 3' end formation in cultured cells. *Mol Cell Biol* 13:3359–3369.
- Nesic D, Maquat LE. 1994. Upstream introns influence the efficiency of final intron removal and RNA 3' end formation. *Genes & Dev* 8:363–375.
- Niwa M, Berget SM. 1991. Mutation of the AAUAAA polyadenylation signal depresses in vitro splicing of proximal but not distal introns. *Genes & Dev* 5:2086–2095.
- Niwa M, Rose SD, Berget SM. 1990. In vitro polyadenylation is stimulated by the presence of an upstream intron. *Genes & Dev* 4:1552–1559.
- O'Connor JP, Alwine JC, Lutz CS. 1997. Identification of a novel, non-snRNP protein complex containing U1A protein. *RNA* 3:1444–1455.
- Patton JG, Porro EB, Galceran J, Tempst P, Nadal-Ginard B. 1993. Cloning and characterization of PSF, a novel pre-mRNA splicing factor. *Genes & Dev* 7:393–406.
- Proudfoot, N. 1996. Ending the message is not so simple. *Cell* 87:779–781.
- Staley JP, Guthrie C. 1998. Mechanical devices of the spliceosome: Motors, clocks, springs, and things. *Cell* 92:315–326.
- Wahle E, Keller W. 1996. The biochemistry of polyadenylation. *TIBS* 21:247–251.