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Identification of a novel, non-snRNP protein complex containing U1A protein

J. PATRICK O'CONNOR,[1,3] JAMES C. ALWINE,[2] and CAROL S. LUTZ[2]

[1]Department of Orthopaedic Surgery, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA
[2]Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA

ABSTRACT

Mouse monoclonal antibodies (MAbs) were generated against *Escherichia coli*-produced U1snRNP-A (U1A) protein. U1A-specific MAbs as well as MAbs that reacted with both U1A and U2snRNP-B″ (U2B″) were isolated. MAb 12E12 was unique among the characterized MAbs because it failed to immunoprecipitate U1A protein produced by in vitro transcription and translation using rabbit reticulocyte lysates. However, when U1A protein was made using a wheat germ extract, MAb 12E12 could immunoprecipitate U1A quite readily, as did the other MAbs. These data suggest that the MAb 12E12 epitope is masked when U1A is prepared in reticulocyte lysate. Further studies showed that MAb 12E12 recognizes an epitope that is masked when U1A protein is bound to U1 RNA. The unique nature of MAb 12E12 was used to demonstrate that U1A could be immunoprecipitated from whole-cell extracts in a form that was free of U1 RNA and other snRNP components. However, this snRNP-free U1A (SF-A) was found to co-immunoprecipitate with a unique set of non-snRNP proteins. In order to confirm that U1A exists in at least two distinct complexes (snRNP bound and snRNP free), [^{35}S]-labeled nucleoplasmic extracts were analyzed by sucrose density gradient fractionation and immunoprecipitation. MAb 12E12 specifically immunoprecipitated SF-A, which migrated in a novel non-snRNP complex. Specifically, proteins of approximately 58, 59, 63, 65, and 105 kDa co-sedimented and co-immunoprecipitated with SF-A. Our data show that a significant portion of the cellular U1A (at least 3% or approximately 30,000 molecules) exists in the nucleoplasm in one or more novel complexes. Our previous studies have demonstrated an effect of purified U1A on polyadenylation of pre-mRNAs and, consistent with this finding, purified antibodies to SF-A significantly diminish polyadenylation in vitro.

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

INTRODUCTION

Vertebrate small nuclear ribonucleoprotein particles (snRNPs) are protein–RNA complexes that are intimately involved in the catalysis of RNA processing reactions. The well-studied U1, U2, U4, U5, and U6 snRNPs have established roles in pre-mRNA splicing (reviewed by Moore et al., 1993), and are quite abundant in the nucleus, ranging from thirty thousand to one million copies per cell, depending on the snRNP (Baserga & Steitz, 1993).

The U1snRNP is composed of the 164-nt U1 RNA, a set of proteins common to all snRNPs (the Sm proteins: B, B′, D, D′, E, F, and G), and three U1snRNP-specific proteins, A, C, and 70K (reviewed in Lührmann et al., 1990). Besides its role in splicing, the U1snRNP, or components of it, also functions in polyadenylation. We have previously demonstrated that the U1snRNP-A protein (U1A) can bind to the SV40 late polyadenylation signal (Lutz & Alwine, 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 analy-

Reprint requests to: James C. Alwine, 560 Clinical Research Building, 415 Curie Blvd., University of Pennsylvania, Philadelphia, Pennsylvania 19104-6142, USA; e-mail: alwinen@mail.med.upenn.edu.
[3]Present address: Department of Orthopaedics, UMDNJ—New Jersey Medical School, Newark, New Jersey 07103, USA.

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ses, the U1A protein was functioning free of other U1snRNP components, i.e., snRNP-free U1A (SF-A).

That SF-A may function in the cell has been suggested by experiments indicating that it mediates a very specific negative feedback regulatory mechanism affecting the polyadenylation of its own mRNA. This involves specific interactions with both the U1A mRNA and poly(A) polymerase (Gunderson et al., 1994, 1997). However, our previous in vitro data suggests that SF-A also mediates a more general, positive effect on polyadenylation. These findings bring to light 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 is based upon the high affinity ($K_d \approx 10^{-9}$) of U1A for U1 RNA (Lutz-Freyermuth et al., 1990; Jessen et al., 1991), as well as the negative regulation of U1A's own polyadenylated message (Boelens et al., 1993).

In order to more quantitatively and qualitatively examine SF-A, we have generated a series of U1A-specific monoclonal antibodies. U1A is a 282-amino acid polypeptide that contains two RNA-binding domains, called RNP motifs (Bandziulis et al., 1989; Dreyfuss et al., 1993; Burd & Dreyfuss, 1994) or RNA recognition motifs (RRMs; Query et al., 1989a; Kenan et al., 1991). These motifs are separated by a central proline-rich sequence in U1A. The amino-terminal RNP motif or RRM binds to the second stem-loop of U1 RNA (Lutz-Freyermuth & Keene, 1989; Lutz-Freyermuth et al., 1990). In this study, we describe the characterization of the anti-U1A monoclonal antibodies and demonstrate 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 [the SF-A complex(es)]. We also demonstrate that SF-A has a physiologically relevant function by showing that anti-SF-A antibodies inhibit in vitro polyadenylation of an SV40 late polyadenylation substrate RNA. This is the first demonstration of an snRNP protein as a component of a relatively abundant non-snRNP complex.

RESULTS

Production of U1A-specific monoclonal antibodies

We have produced several murine monoclonal antibodies (MAbs) that recognize human U1A protein. U1A protein was expressed and purified from *Escherichia coli* to immunize BALB/c mice (see Materials and Methods). Splenocytes from one mouse were fused to the murine myeloma cell line SP2/O and hybridomas were selected in hypoxanthine–azaserine-containing media. Hybridomas secreting anti-U1A antibody were selected by ELISA and western blot analyses. Of approximately 1,400 hybridomas screened, nine hybridomas were selected. Six hybridomas secreted antibodies that specifically recognized U1A and three secreted antibodies that also recognized a protein which co-migrated with U2B″, a structurally similar U2snRNP-specific protein. Four of the hybridomas were chosen for further analysis. Western blot analyses of HeLa cell total extracts (Fig. 1) demonstrated that MAbs 1E1, 10E3, and 12E12 specifically recognize a 32-kDa protein, the appropriate size for U1A, whereas MAb 20F8 recognizes two proteins of 32 and 28 kDa, the appropriate sizes for U1A and U2B″. In contrast, nonspecific mouse polyclonal antibodies (SP2/O ascites fluid) failed to react strongly to any HeLa cell proteins. Control MAb Y12, previously characterized for its specificity to the Sm complex (Lerner et al., 1981), specifically recognized two proteins of approximately 25 and 26 kDa, the expected sizes of the Sm proteins B and B′. These results suggest that MAbs 1E1, 10E3, and 12E12 specifically

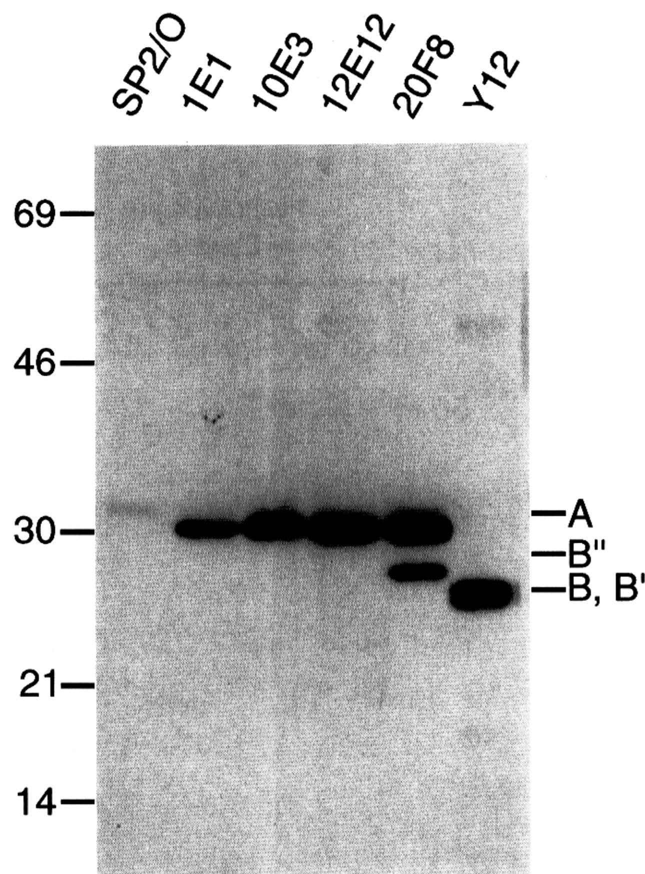

FIGURE 1. Western blot analysis of different U1A MAbs. HeLa total cell extract was separated by electrophoresis on a 12.5% SDS-PAGE gel. The gel was then transferred to nitrocellulose and the blot was cut into strips. Each individual strip was probed with the MAb listed above each lane, and the reactive bands were visualized by chemiluminescence (ECL, Amersham). SP2/0, negative control; 1E1, 10E3, 12E12, U1A reactive MAbs; 20F8, U1A/U2B″ reactive MAb; Y12, Sm control MAb. Positions of U1A, U2B″, and Sm B/B′ proteins are marked at right.

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react with U1A, whereas MAb 20F8 recognizes both U1A and U2B″.

Immunoprecipitation of U1A protein made in vitro

To confirm that the MAbs are binding to U1A, we tested for the ability of the MAbs to immunoprecipitate U1A protein made in vitro (Fig. 2). [^{35}S]-methionine labeled-U1A protein was prepared using a coupled transcription/translation system that employed either rabbit reticulocyte lysate or a wheat germ extract. MAbs 1E1, 10E3, and 20F8 readily immunoprecipitated U1A protein prepared in reticulocyte lysates, however, MAb 12E12 failed to do so, as did SP2/0 ascites fluid, the negative control (Fig. 2, left panel). The largest of the three prominent U1A bands seen in the reticulocyte lysate translation appears to be full length, whereas the smaller two are breakdown products. Interestingly, we found that all of the MAbs, including MAb 12E12, could immunoprecipitate U1A protein prepared in a wheat germ extract (Fig. 2, right panel).

These results confirm that MAbs 1E1, 10E3, 12E12, and 20F8 recognize U1A protein. However, the data suggest that MAb 12E12 is unusual in that its epitope on U1A is masked when the protein is produced in reticulocyte lysates. Epitope mapping experiments, using bacterially prepared fragments of U1A, indicated that the MAb 12E12 epitope is located in the amino-terminal half of U1A, whereas the epitopes for the other three MAbs are located in the carboxy-terminal half of U1A (data not shown).

We have also demonstrated that all the MAbs can recognize U1A protein made in bacteria by both ELISA and western blot analysis (data not shown). We conclude that MAbs 1E1, 10E3, and 12E12 specifically recognize U1A. We also conclude that MAb 20F8 recognizes both U1A and a 28-kDa protein that is most likely U2B″, based upon the known gel mobility of U2B″, its primary sequence similarity to U1A, and the immunofluorescence staining pattern of MAb 20F8 (see below, Fig. 3). MAbs 1E1, 10E3, and 12E12 were determined to be $IgG_1\kappa$ subtypes and MAb 20F8 was found to be an $IgG_1\lambda$ subtype (data not shown).

Immunofluorescence analysis using the MAbs

Immunofluorescence analysis (Fig. 3) demonstrated that MAbs 1E1 and 10E3 gave a uniform nuclear distribution that excluded the nucleoli (Fig. 3A,B), although

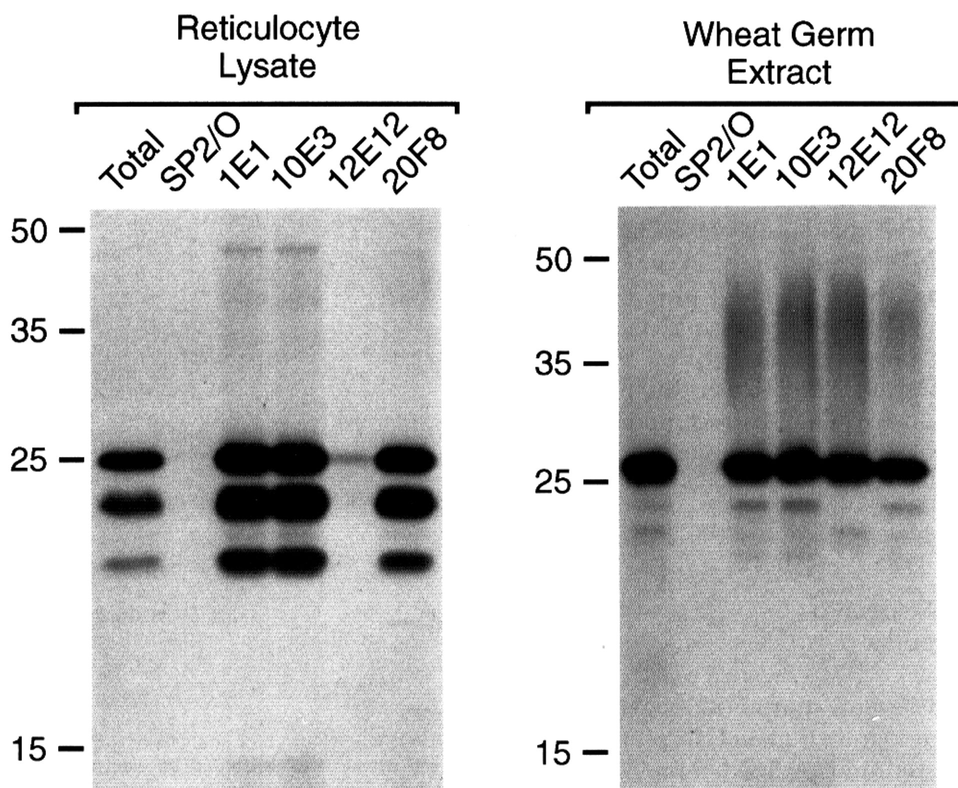

FIGURE 2. MAb 12E12 can immunoprecipitate U1A prepared in wheat germ extract but not U1A prepared in reticulocyte lysate. [^{35}S]methionine-labeled U1A protein was prepared by in vitro transcription/translation in both rabbit reticulocyte lysates (left) and wheat germ extracts (right). Immunoprecipitations were then performed using the MAbs listed above each lane, and the immunoprecipitates were analyzed on a 12.5% SDS-PAGE gel. Total, 1/10 input; SP2/0, negative control. Molecular weight markers (Perfect Protein Markers, Novagen) were found to migrate slightly aberrantly in this gel system.

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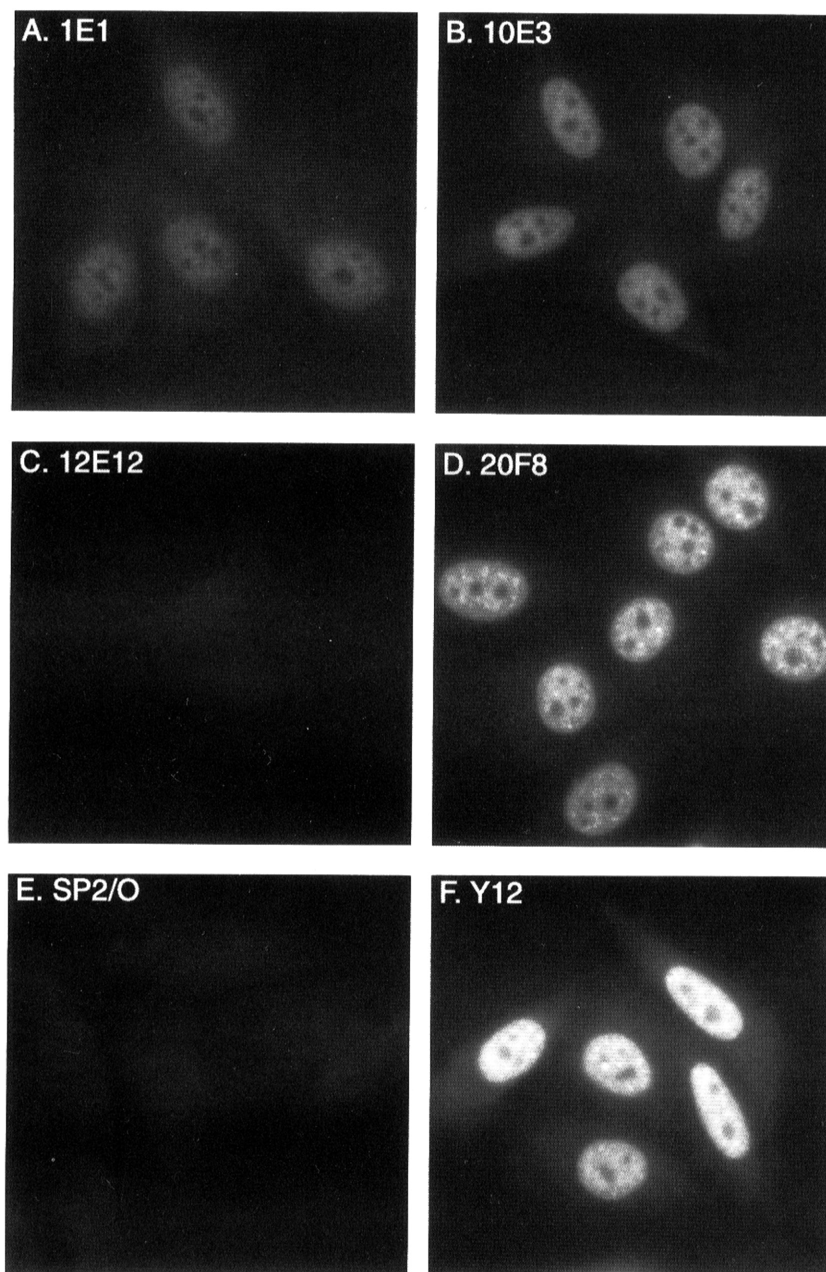

FIGURE 3. Immunofluorescence of HeLa cells reveals differences between the U1A MAbs. HeLa cells were grown on cover slips, fixed in paraformaldehyde, and permeabilized with Triton X-100. Antibodies used are indicated in the upper left-hand corner of each photograph. **A,B,C:** U1A reactive MAbs. **D:** U1A/U2B″ reactive MAb. **E:** SP2/0, negative control. **F:** Y12, Sm control.

MAb 1E1 gave a consistently weaker immunofluorescence signal. This staining pattern is consistent with the uniform nonnucleolar distribution of U1snRNPs as indicated by in situ hybridization to U1 RNA (Carmo-Fonseca et al., 1991). MAb 20F8 also gave a predominant nuclear signal that excluded the nucleoli; however, in addition, several small concentrations of antigen or speckles were noted (Fig. 3D). The staining pattern of MAb 20F8 was very similar to that of MAb Y12, which recognizes the Sm proteins B and B′ (Fig. 3F). This pattern is consistent with both the distribution of U1snRNPs and the speckled distribution of U2snRNPs as indicated by in situ hybridization to U1 and U2 RNAs (Carmo-Fonseca et al., 1991). These data support the above results, suggesting that MAb 20F8 recognizes both U1A and U2B″. Interestingly, MAb 12E12 showed no more immunofluorescence than the negative control, SP2/O ascites fluid (compare Fig. 3C and E). This finding correlates with the data in Figure 2, suggesting that conditions exist where the MAb 12E12 epitope may be masked. Alternatively, MAb 12E12 may be nonfunctional in immunofluorescence analyses.

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Immunoprecipitation of U1snRNPs from human cells

We next determined if the MAbs could immunoprecipitate U1snRNPs from human cells. Nucleoplasm was prepared from human 293T cells labeled in vivo with [^{35}S]-methionine and [^{35}S]-cysteine in a 1% Triton X-100 buffer as described previously (Piñol-Roma et al., 1990). A total cell extract was also prepared from labeled 293T cells using buffer containing RIPA detergents. RIPA detergents should disrupt protein–protein interactions, thereby permitting a comparison between the Triton X-100 extract and the RIPA extract for the suggestion of interacting proteins. As seen in Figure 4, the control SP2/O ascites fluid failed to immunoprecipitate any proteins from nucleoplasm (lane 1), although some proteins could be detected in the total extract immunoprecipitation (lane 2). However, these pro-

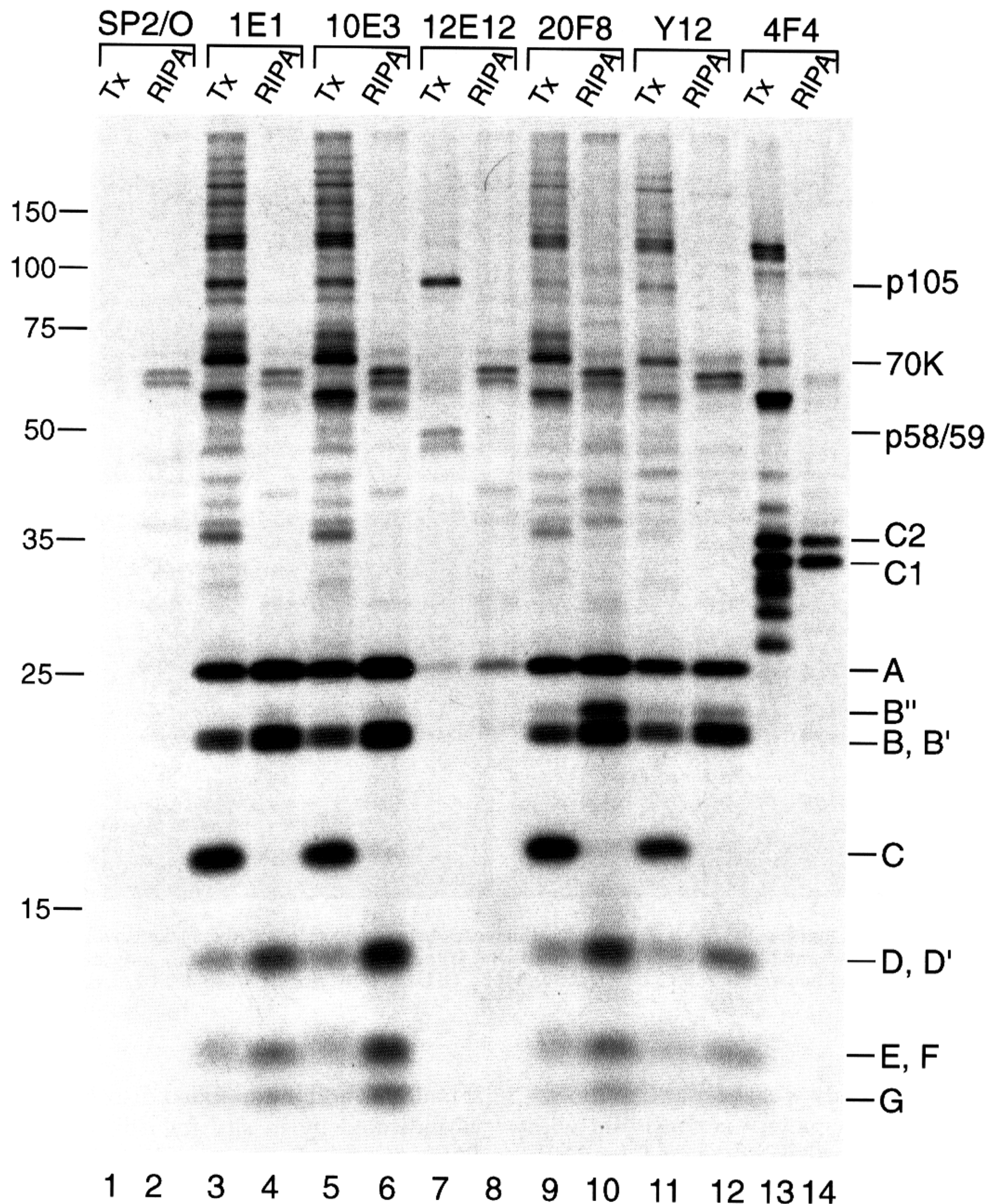

FIGURE 4. Immunoprecipitation of complexes from [^{35}S]methionine-labeled human 293T cellular extracts. Nucleoplasm was prepared from metabolically labeled 293T cells in RSB buffer (100 mM NaCl, 10 mM Tris, pH 7.5, 2.5 mM $MgCl_2$) including 1% Triton X-100 (odd-numbered or Tx lanes). Total 293T cell extract was prepared in the same RSB buffer containing 1% Triton, 1% deoxycholate, and 0.1% SDS (even-numbered or RIPA lanes). Immunoprecipitations were then performed using the antibodies listed above the lanes and the resulting complexes were washed in the appropriate buffers. Identifiable proteins are marked on the right. SP2/0, negative control; 1E1, 10E3, 12E12, U1A-reactive MAbs; 20F8, U1A/U2B''-reactive MAb; Y12, Sm control, 4F4, hnRNP control. Molecular weight markers (Perfect Protein Markers, Novagen) on this gel migrated slightly aberrantly.

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teins appear to bind nonspecifically because they appear in all the RIPA total extract immunoprecipitates except the MAb 4F4 control (Fig. 4, even-numbered lanes).

We used two positive controls, MAb 4F4, which recognizes hnRNP proteins C1 and C2 (Dreyfuss et al., 1984), and MAb Y12, described above (Lerner et al., 1981). As expected, MAb 4F4 immunoprecipitated a characteristic hnRNP complex from nucleoplasm (Fig. 4, lane 13), but only immunoprecipitated hnRNP C1 and C2 from the RIPA total extract (lane 14). These results indicate that our extracts and immunoprecipitation conditions are adequate for detecting ribonucleoprotein complexes and protein-protein interactions. MAb Y12 immunoprecipitated several proteins from nucleoplasm with molecular weights characteristic of the Sm proteins (B, B′, D, D′, E, F, and G), the U1snRNP-specific 70K, A, and C proteins, the U2snRNP-specific B″ protein, and other predominantly higher molecular weight proteins that we have not characterized (Fig. 4, lane 11). In contrast, MAb Y12 immunoprecipitated U1A, U2B″, and the Sm proteins from the RIPA total extract, but not the U1snRNP-specific 70K and C proteins nor the higher molecular weight proteins (Fig. 4, lane 12). The results indicate that the RIPA detergent treatment fails to disrupt U1A or the Sm proteins from binding to U1 RNA, whereas it can disrupt 70K and C binding to the U1snRNP. These two proteins interact with the U1snRNP through both protein–RNA and protein-protein interactions (Query et al., 1989b; Nelissen et al., 1991, 1994). The complement of proteins immunoprecipitated by MAbs 1E1, 10E3, and 20F8 from both nucleoplasm and RIPA total extract were very similar to those immunoprecipitated by MAb Y12 (Fig. 4, lanes 3–6, 9, and 10). These results clearly indicate that MAbs 1E1, 10E3, and 20F8 can immunoprecipitate the U1snRNP. We could detect some differences in the complement of proteins immunoprecipitated by MAbs 1E1, 10E3, and 20F8 versus those immunoprecipitated by MAb Y12. Notably, a protein migrating at the same position as hnRNP C2 and another protein migrating just above 70K protein (at the 75-kDa molecular weight marker in Fig. 4) are present in the nucleoplasm immunoprecipitates of MAb 1E1, 10E3, and 20F8, but not in the MAb Y12 immunoprecipitate (compare lanes 3, 5, 9, and 11 of Fig. 4). By comparing the nucleoplasm and RIPA total extract immunoprecipitates of MAb 20F8, we also detected an increase in the amount of U2B″ immunoprecipitated in the RIPA total extract, suggesting that, in a native U2snRNP, the MAb 20F8 epitope is partially masked on U2B″ (compare lanes 9 and 10 of Fig. 4).

Unexpectedly, we found that MAb 12E12 immunoprecipitated a smaller amount of U1A protein from nucleoplasm and this was not accompanied by the other characteristic snRNP proteins (Fig. 4, lanes 7 and 8). In addition to U1A, MAb 12E12 immunoprecipitated three other prominent proteins from nucleoplasm. These included proteins with an apparent mass of 105 kDa (p105), a doublet of proteins migrating at approximately 59 and 58 kDa (p59 and p58), and U1A. In contrast, when RIPA total-cell extract was immunoprecipitated with MAb 12E12, the only specific protein immunoprecipitated was U1A. These results indicate that a fraction of U1A is not associated with the U1snRNP, but may be associated with other non-snRNP proteins. By exhaustive immunoprecipitation of [^{35}S]-labeled nucleoplasm and quantitation using a phosphorimager, we estimated that as much as 3% of the total U1A present in nucleoplasm could be immunoprecipitated with MAb 12E12 (data not shown). We also noted that proteins with the same apparent gel mobility as p105 and p58 were present in the MAb 1E1 and 10E3 immunoprecipitates (Fig. 4, lanes 3 and 5), suggesting that these MAbs may be able to recognize not only U1A complexed with snRNP, but also the fraction of U1A that is apparently snRNP-free.

MAb 12E12 precipitates U1A that is not bound to U1 RNA

To confirm the observation that MAb 12E12 recognizes a fraction of U1A that is not associated with the U1snRNP, we examined immunoprecipitates by western and northern blot analysis (Fig. 5). Immunoprecipitations were performed on unlabeled 293T cell nucleoplasm extracts using the various MAbs, then each immunoprecipitate was split in half for either western or northern blot analysis. Because MAb 12E12 appeared to immunoprecipitate less U1A than the other U1A-specific MAbs, five independent immunoprecipitates were pooled to produce the sample for MAb 12E12 in Figure 5. Rabbit anti-U1A polyclonal sera was used to detect U1A and U2B″ in the western blot and, as expected, U1A was found in all the immunoprecipitates except the negative control, SP2/O (Fig. 5, top). U2B″ was also detected in the MAb 20F8 and Y12 immunoprecipitates, but not in the MAb 1E1, 10E3, or 12E12 immunoprecipitates, in agreement with the data in Figures 1, 3, and 4.

Northern blot analysis of the immunoprecipitates using a [^{32}P]-labeled oligonucleotide probe for U1 RNA clearly demonstrated that U1 RNA was present in the MAb 1E1, 10E3, 20F8, and Y12 immunoprecipitates, confirming the data in Figure 4, and thus indicate that these antibodies could precipitate U1A associated with the U1snRNP. MAb 12E12 precipitated no U1 RNA, also confirming the data in Figure 4, which indicated that MAb 12E12 immunoprecipitates U1A that is not associated with the U1snRNP. We have termed the fraction of U1A that is not associated with the U1snRNP as SF-A and the complex of proteins immunoprecipi-

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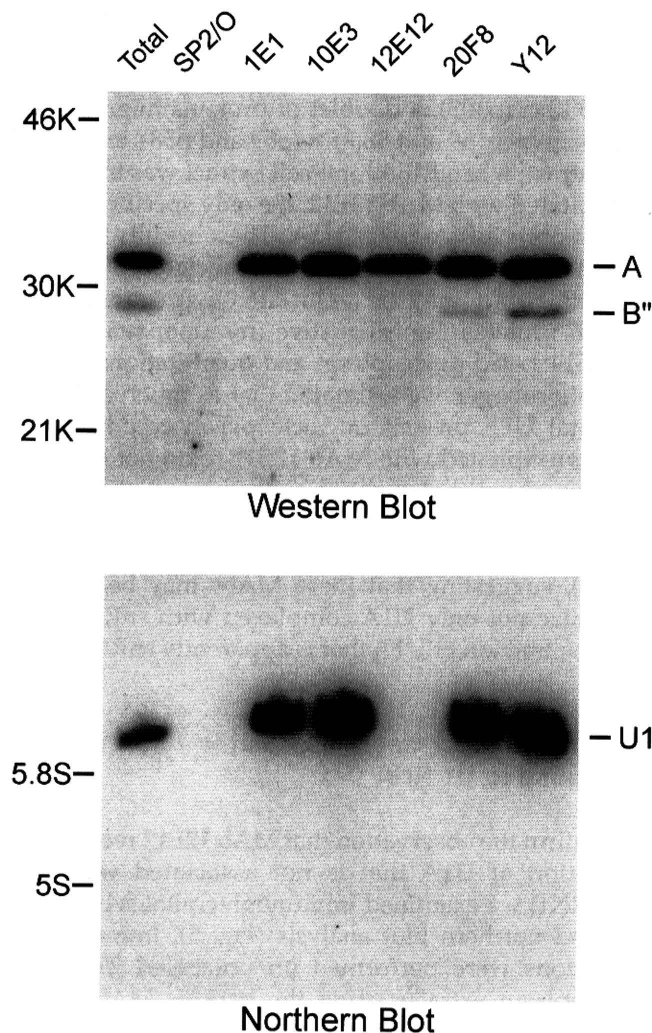

FIGURE 5. Immunoprecipitation of 293T cellular extracts followed by western and northern blotting reveals non-U1snRNP-associated U1A protein. Immunoprecipitations were performed on unlabeled 293T cellular extracts using the antibodies listed at the top of the figure. The immunoprecipitates were then split in half to be used on the western and northern blots. The 12E12 lanes represent five separate immunoprecipitations pooled together. Top: Western blot was probed using a rabbit anti-U1A polyclonal antibody (Lutz & Alwine, 1994) and the reactive bands were visualized by [^{125}I]-labeled donkey anti-rabbit secondary antibody. Position of U1A and U2B″ are marked on the right. Bottom: Northern blot was probed with a [^{32}P]-labeled oligonucleotide complementary to the 5′-end of U1 RNA. Position of U1 RNA is marked on the right; positions of 5.8S and 5S rRNA are indicated on the left.

tated with MAb 12E12 (U1A, p105, p58, p59) as the SF-A complex(es).

One explanation for the specificity of MAb 12E12 for SF-A is that the epitope may be masked when the protein is bound to U1 RNA. This would be supported by (1) the above data, which maps the epitope to the amino-terminal portion of U1A protein, containing the U1 RNA binding site; and (2) the inability of MAb 12E12 to precipitate U1A prepared in reticulocyte lysate, suggesting that a protein or nucleic acid present in the reticulocyte lysate may mask the epitope. In order to test this hypothesis, we examined the effects of adding in vitro-transcribed U1 RNA to [^{35}S]-methionine-labeled U1A protein, made by in vitro transcription and translation using a wheat germ extract, and then determined the ability of MAb 12E12 to immunoprecipitate U1A. Figure 6 shows that the addition of increasing amounts of U1 RNA (10–1,000 ng) to the wheat germ-produced U1A had no deleterious effect on the ability of MAb 1E1 to immunoprecipitate U1A (Fig. 6, lanes 5–8). In contrast, the same amounts of U1 RNA dramatically inhibited the ability of MAb 12E12 to immunoprecipitate U1A (Fig. 6, lanes 9–12). Negligible amounts of U1A were immunoprecipitated with SP2/O under all treatments (Fig. 6, lanes 1–4). These data suggest that the MAb 12E12 epitope is masked when U1A binds to U1 RNA and provide an explanation as to why MAb 12E12 preferentially immunoprecipitates U1A not associated with the U1snRNP.

Analysis of the SF-A complexes

The data in Figure 4 indicate that SF-A is complexed with at least three other proteins, p105, p59, and p58. In order to establish that this was a true complex distinct from the U1snRNP, we performed sucrose-density gradient centrifugation of [^{35}S]-methionine and [^{35}S]-cysteine-labeled nucleoplasm. Thirty fractions were collected and an equal portion of each fraction was immunoprecipitated with either MAb 10E3 or 12E12. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. Figure 7 shows that the U1snRNP predominantly sedimented in fractions 17–23 (approximately 11S; Lerner et al., 1980), based upon the presence of U1A, the U1snRNP-specific proteins C and 70K, and the snRNP core proteins B, B′, D, and D′ (Fig. 7A). In contrast, the SF-A complex sedimented in fractions 7–11 (approximately 3–4S), based upon the presence of U1A, p58, p59, and p105 and the absence of U1snRNP proteins other than U1A (Fig. 7B). MAb 10E3 immunoprecipitated both the U1A associated with the snRNP and with the SF-A complex, suggesting that this epitope on U1A is available in both fractions. MAb 12E12 preferentially precipitated the SF-A complex(es), in agreement with the previous data, suggesting that this MAb is specific for SF-A and that its epitope is masked when U1A is associated with the snRNP. These results demonstrate that the SF-A complex is genuine and distinct from the U1snRNP, that it can be separated physically, and that two different monoclonal antibodies, recognizing different epitopes on U1A, can immunoprecipitate SF-A complex(es).

The SF-A complex(es) detected in the gradient analysis are better defined than in the data shown in Figure 4. Examination of the gradient fractionation suggests that at least two more proteins may be in the SF-A complex(es), and these proteins have apparent

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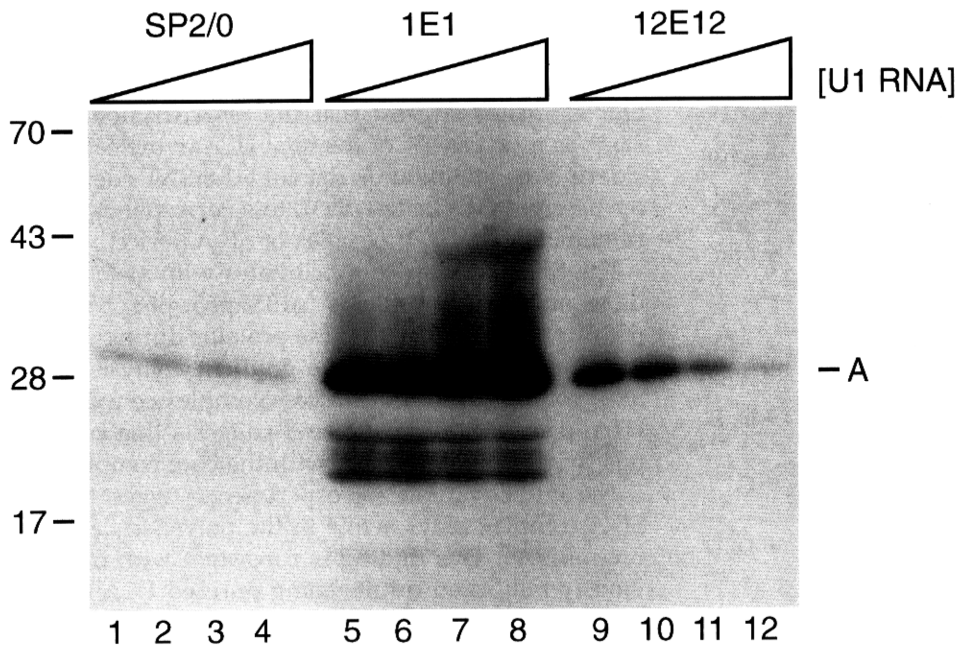

FIGURE 6. Exogenous U1 RNA masks the 12E12 epitope on U1A protein. [^{35}S]-labeled U1A protein was prepared by in vitro transcription/translation in wheat germ extracts and was incubated with unlabeled in vitro-transcribed U1 RNA in increasing amounts. The pre-bound complexes were then added to negative (SP2/0) or to U1A-reactive (1E1 or 12E12) MAbs and immunoprecipitations were performed. Lanes 1, 5, 9, no exogenous U1 RNA; lanes 2, 6, 10, 10 ng U1 RNA; lanes 3, 7, 11, 100 ng U1 RNA; lanes 4, 8, 12, 1,000 ng U1 RNA.

molecular weights of approximately 63 and 65 kDa (p63 and p65 respectively, Fig. 7B). Therefore, at least five proteins appear to be able to complex with U1A outside the U1snRNP context. No RNA species appear to be associated with the SF-A complex based upon immunoprecipitation analysis of [^{32}P]-orthophosphate labeled cell extracts (data not shown).

Perhaps most intriguing were the differences in protein content of the SF-A complexes recognized by the different MAbs and between different gradient fractions. Whereas MAb 12E12 could immunoprecipitate SF-A complexes containing all of the proteins (U1A, p105, p65, p63, p59, and p58), MAb 10E3 immunoprecipitated complexes containing only U1A, p58, p65, and p105 (compare fraction 9, Fig. 7A,B). Among the SF-A complexes recognized by MAb 12E12, p59 was more abundant in fraction 7 and absent in fraction 11, whereas p63 was most abundant in fraction 9 (Fig. 7B). The relative amounts of p58, p65, and p105 in the SF-A complex appeared to be more constant. One possible interpretation of these observations would be that a basal SF-A complex consists of U1A, p58, p65, and p105, and that p59 and p63 dynamically associate with the basal SF-A complex.

Polyadenylation inhibition experiments

Our previous studies using purified recombinant U1A protein had demonstrated that U1A alone had a significant positive effect on polyadenylation (Lutz et al., 1996). In order to determine whether similar functions were mediated by SF-A, we asked whether anti-SF-A antibodies would significantly inhibit an in vitro polyadenylation reaction. Increasing amounts of purified SP2/0, 10E3, or 12E12 antibodies were added to in vitro polyadenylation reactions using a substrate RNA containing the SV40 late polyadenylation signal (Fig. 8). Increasing amounts of purified SP2/0, the negative control, had no effect on polyadenylation; however, increasing amounts of purified 12E12 significantly diminished polyadenylation of the substrate RNA. These data suggest a physiologically relevant function for SF-A in polyadenylation reactions. At high levels, purified 10E3 mediated a slight inhibition of polyadenylation; this is likely due to the fact that 10E3 precipitates small amounts of SF-A complexes (see Fig. 7). Similar results were obtained using a substrate RNA that represents the HIV polyadenylation signal (data not shown), suggesting that the effect of SF-A on polyadenylation is not limited to the SV40 late polyadenylation signal.

DISCUSSION

We have described a number of MAbs that recognize the U1A protein. One of these, MAb 12E12, is of particular interest because it recognizes an epitope that is masked when U1A is part of the U1snRNP, thus it detects an SF-A fraction. Our data indicate that the failure of MAb 12E12 to recognize snRNP-bound U1A is because the epitope for the MAb is within or near the U1 RNA binding site located on the amino-terminal half of U1A. Thus, the epitope appears to be masked when U1A is bound to the snRNP. The other MAbs tested (1E1, 10E3, 20F8) recognize epitopes on the carboxy-terminal half of U1A and, therefore, precipitate the snRNP-associated U1A as well as SF-A.

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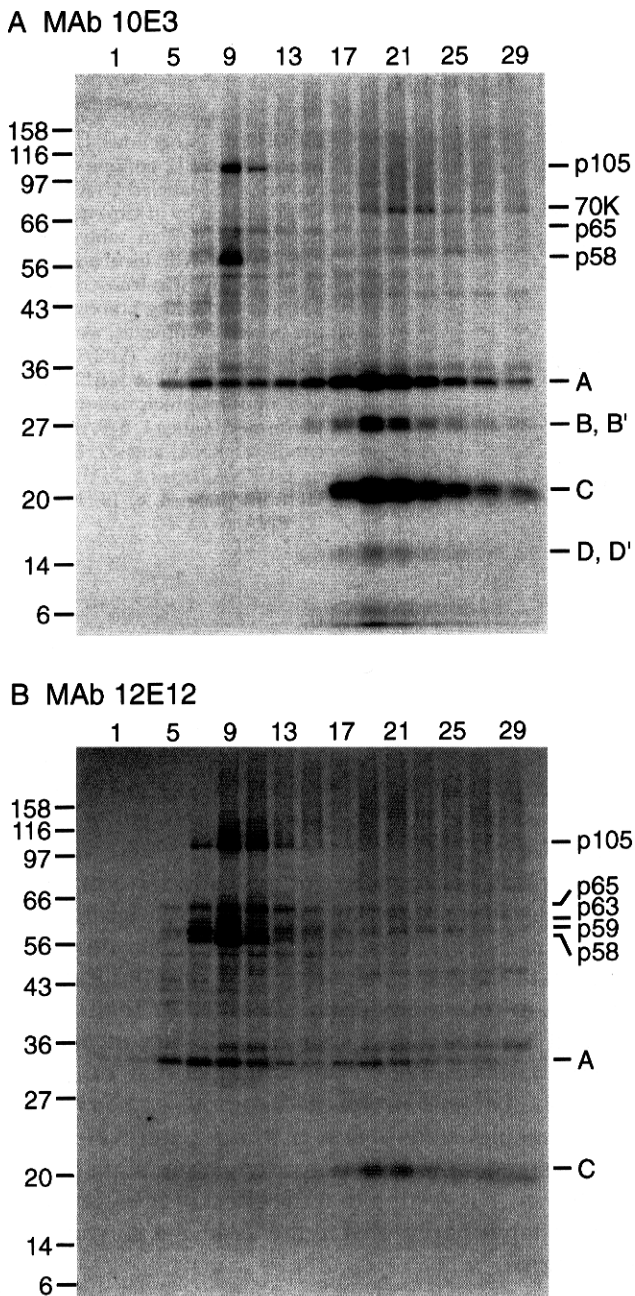

FIGURE 7. Sucrose gradient analysis followed by immunoprecipitation reveals distinct, novel complexes are precipitated by MAb12E12. 293T cells were metabolically labeled with [^{35}S]-methionine and -cysteine and nucleoplasm was prepared as described in Materials and Methods. Nucleoplasm was applied to the top of a 5–30% sucrose gradient and was centrifuged at 25,000 RPM for 42 h in an SW28 rotor. One-milliliter fractions were collected and were split in half for immunoprecipitations by either MAb 10E3 (**A**) or 12E12 (**B**). Every other fraction (odd numbers) was analyzed. Fraction 1 represents the top of the gradient. Molecular weight markers are indicated on the left side; proteins are identified on the right.

Previous data have suggested that the tight binding of U1A protein to U1 RNA, plus the apparent similar intracellular amounts of U1A and U1 RNA, may result in there being little or no U1A free of the snRNP. Also, the inhibitory interaction of U1A with poly(A) polymerase suggested a mechanism for autoregulation of U1A's own mRNA levels, thus limiting the amount of free U1A (Boelens et al., 1993; Gunderson et al., 1994). Our estimates suggest that the SF-A fraction represents as much as 3% of the total U1A in the cell. Considering the quantitation data of U1snRNPs described by Baserga and Steitz (1993), this corresponds to approximately 30,000 molecules of SF-A per cell.

The SF-A fraction co-precipitates with at least five other non-snRNP proteins (p105, p65, p63, p59, and p58), and migrates with these proteins during sucrose gradient centrifugation. The gradient analysis of the SF-A complexes indicates a core complex consisting of U1A, p58, p65, and p105, and suggests that p59 and p63 dynamically associate with the core complex.

The antibody inhibition experiments suggest that the SF-A complex plays a role in the polyadenylation of pre-mRNAs. This finding is consistent with our previously published results using purified U1A protein (Lutz et al., 1996). However, we do not rule out the possibility that U1A as part of the U1snRNP may also affect RNA processing.

In summary, our data establishes that U1A protein is not an exclusive component of the U1snRNP. A significant fraction is found in a snRNP-free form in complex(es) with other cellular proteins. The effect of the anti-SF-A antibodies on in vitro polyadenylation suggests that the snRNP-free form of U1A is physiologically relevant and is involved in RNA processing.

MATERIALS AND METHODS

Recombinant U1A purification

U1A was purified from *E. coli* BL21(DE3) transformed with pET:g10A (Lutz-Freyermuth et al., 1990) as described previously (Lutz & Alwine, 1994). Briefly, the polyethylenimine precipitate of the bacterial lysate was extracted by sonicating the pellets in HSTE buffer (1 M NaCl, 20 mM Tris-Cl, pH 8, 2 mM Na_2EDTA). Insoluble material was removed by centrifugation. The HSTE soluble fraction was then ammonium sulfate precipitated (30 g ammonium sulfate per 100 mL HSTE fraction). The ammonium sulfate precipitate was collected by centrifugation, resuspended in 20 mM Tris-Cl, pH 8, and then dialyzed extensively against NH8 buffer (100 mM NaCl, 20 mM HEPES, pH 8). After removing insoluble material by centrifugation, the dialyzed fraction was applied to BIOREX 70 column that had been equilibrated in NH8 buffer. The column was developed with a linear salt gradient from 0.1 to 0.6 M NaCl in 20 mM HEPES, pH 8. Column fractions were assayed for purity by SDS-PAGE and Coomassie blue staining and for RNase contamination by challenging [^{32}P]-labeled RNA with fraction aliquots. Pure, RNase-free fractions were pooled.

Monoclonal antibody production

Purified recombinant U1A protein emulsified in Freund's adjuvant was used to immunize BALB/c mice by intraper-

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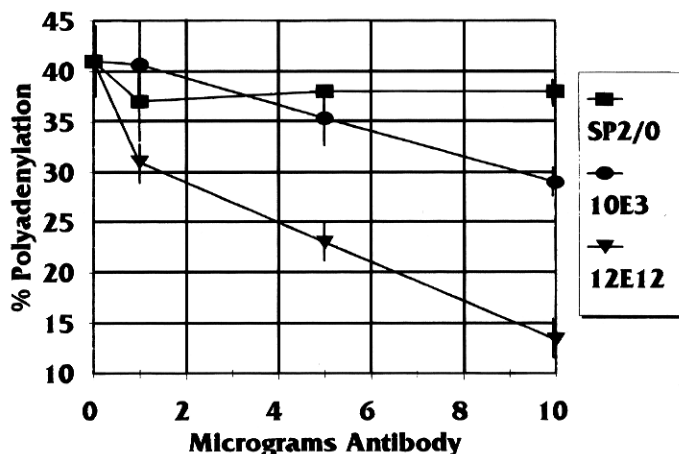

FIGURE 8. Anti-SF-A antibodies inhibit SV40 late polyadenylation in vitro. In vitro polyadenylation reactions were performed using HeLa nuclear extract, a substrate RNA representing the SV40 late polyadenylation signal, cordycepin 5′ triphosphate, and increasing amounts of purified monoclonal antibodies (see Materials and Methods). Percent polyadenylation was measured as percent cleavage divided by total counts present in the reaction. SP2/0 (negative control) data points represent an average of two separate experiments; 10E3 and 12E12 data points represent an average of three separate experiments. Error bars are indicated.

itoneal injection. Hybridoma selection and screening and ascites fluid production were performed as described previously (Choi & Dreyfuss, 1984). Positive hybridomas were subsequently confirmed by western blot analyses of *E. coli* extract containing U1A and HeLa cell total extract. Antibody isotypes were determined with an isotyping kit (Boehringer Mannheim).

Cell culture and labeling

Human 293T and HeLaJW36 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 atmosphere. For in vivo labeling, 10-cm dishes of 293T cells at 50–70% confluency were first washed with phosphate-buffered saline (PBS) and then labeled for 4–6 h in 3 mL of DMEM lacking methionine, but supplemented with 5% fetal calf serum and 50 μCi/mL [^{35}S]-methionine (Amersham, AG1094) or 100 μCi/mL of a mixture of [^{35}S]-methionine and [^{35}S]-cysteine (Amersham, SJQ0079).

Immunoprecipitation

Immunoprecipitations were performed as described previously (Piñol-Roma et al., 1990). Nucleoplasm and total cell extract were prepared from 293T cells using RSB100 (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 1 mM $MgCl_2$) containing 1% Triton X-100 (RSB100-Tx) or RIPA detergents (1% Triton X-100, 1% deoxycholate, and 0.1% SDS; RSB100-RIPA), respectively. Aliquots of nucleoplasm or total extract were incubated with antibody-protein G beads (5 μL ascites fluid per 25 μL GammaBind Plus Sepharose, Pharmacia) for 30–60 min at 4 °C, washed extensively with RSB100-Tx or RSB100-RIPA as appropriate, and then eluted with SDS-PAGE sample buffer.

In vitro transcription and translation

We used coupled T7 RNA polymerase transcription/translation systems that employed either rabbit reticulocyte lysate or wheat germ extract (TNT systems, Promega) and [^{35}S]-methionine (Amersham, AG1094 or SJ1515) to prepare [^{35}S]-labeled U1A protein as per the manufacturer's instructions. A subclone derivative of pGEM2:U1A (Lutz et al., 1996) called YEpC310-U1A was used as the template. Aliquots from each reaction were diluted in RSB100-Tx buffer and immunoprecipitated as described above.

Gel electrophoresis

Proteins were routinely separated on 12.5% SDS-polyacrylamide gels. Gels to be fluorographed were briefly stained with Coomassie blue to identify protein size standards, dehydrated in dimethylsulfoxide (DMSO), impregnated with 22% 2,5-diphenyloxazole (PPO) in DMSO, washed in running water to remove the DMSO and precipitate the PPO, then dried. Fluorography was then performed using Kodak Lightning-plus intensifying screens and either Kodak XAR-5 or Amersham Hyperfilm-MP film at −70 °C. RNA was separated by electrophoresis through 7.5% polyacrylamide gels (29:1 acrylamide:bis-acrylamide) containing 8 M urea in 1× TBE buffer.

Western and northern blots

Proteins were electrophoretically transferred from SDS-PAGE gels onto nitrocellulose, and the membrane was subsequently blocked in PBS containing 5% nonfat dry milk. Primary antibody incubations were performed for 1 h at room temperature. Secondary antibody incubations were performed for 30–60 min at room temperature. Membranes were washed at least three times for 10 min in PBS after each antibody incubation. For primary antibodies, hybridoma culture supernatants were used neat, hybridoma ascites fluid was diluted 1:500 to 1:1,000 in PBS containing 3% bovine serum albumin (PBS-BSA), and rabbit polyclonal anti-U1A sera (Lutz & Alwine, 1994) was diluted 1:500 in PBS-BSA. Monoclonal antibodies were detected by chemiluminescence (ECL system, Amersham) using a horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG, Cappel) diluted 1:10,000 in PBS-BSA and Amersham Hyperfilm-ECL. Rabbit antibodies were detected with [^{125}I]-conjugated donkey anti-rabbit IgG fraction (Amersham, IM1340) diluted to 2 μCi/mL in PBS containing 5% nonfat milk and subsequent autoradiography. The amount of bound [^{125}I]-conjugated antibody was quantitated using a Molecular Dynamics PhosphorImager.

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Northern blots were performed as described previously (Alwine et al., 1977) with modifications by O'Connor and Peebles (1991), except that OHyb contained 100 μg/mL yeast RNA instead of calf liver RNA. U1 RNA was detected with a [^{32}P]-labeled oligonucleotide (POC74, 5'-ATC TCC CCT GCC AGG TAA GTA T-3') complementary to the 5' end of U1 RNA. The amount of bound [^{32}P]-labeled oligonucleotide was quantitated with a Molecular Dynamics PhosphorImager.

Immunofluorescence

HeLaJW36 cells were grown on glass microscope slides or coverslips and fixed with 4% paraformaldehyde in PBS for 10 min. All operations were performed at room temperature. The fixed cells were subsequently permeablized with 1% Triton X-100 in PBS for 5–10 min and then blocked with 3% BSA and 1% goat serum in PBS (PAGS) for at least 15 min. Subsequent primary and secondary antibody incubations were for 60 and 30 min, respectively. After each antibody treatment, slides were washed at least three times with PBS. For primary antibodies, hybridoma culture supernatants were used neat or ascites fluid was diluted 1:100 in PAGS except for Y12 ascites fluid, which was diluted 1:1,000 in PAGS. Secondary antibody was BODIPY-FL conjugated goat anti-mouse IgG (Molecular Probes). Slides were counterstained with DAPI (1 μg/mL) to determine cell stages. Only interphase cells were photographed.

Sucrose gradients

Sucrose gradients (5–30%, w/v) were prepared in RSB100-Tx using a BioComp (New Brunswick, Canada) model 106 gradient master. Gradients were centrifuged in a Beckman SW28 rotor at 25,000 RPM for 42 h at 4 °C. One-milliliter fractions were taken with a BioComp model 150 gradient fractionator and were immunoprecipitated as described above.

Polyadenylation reactions

Polyadenylation reactions were performed using HeLa nuclear extract as described by Schek et al. (1992) with an SV40 late polyadenylation substrate RNA (UPAS, Cooke & Alwine, 1996). Antibodies were purified on Protein G agarose and were dialyzed against buffer D (20 mM Hepes pH 7.6, 20% glycerol, 100 mM KCl, 0.2 mM EDTA). Resulting antibody concentrations were determined by Bradford assays. Reactions also contained 1 mM cordycepin 5' triphosphate (Sigma) and 250 mM ATP to quantitatively measure cleavage without subsequent polyadenylation. Reactions were assembled with purified antibodies and were incubated on ice for 15 min prior to the addition of 1×10^5 cpm RNA per reaction. Reactions were allowed to proceed for 1 h at 30 °C. Percent polyadenylation was measured as percent cleavage divided by total radioactive counts as analyzed on a PhosphorImager.

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