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The nuclear matrix phosphoprotein p255 associates with splicing complexes as part of the [U4/U6.U5] tri-snRNP particle

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ABSTRACT

The monoclonal antibody CC3 recognizes a phosphorylated epitope present on an interphase protein of 255 kDa. Previous work has shown that p255 is localized mainly to nuclear speckles and remains associated with the nuclear matrix scaffold following extraction with non-ionic detergents, nucleases and high salt. The association of p255 with splicing complexes is suggested by the finding that mAb CC3 can inhibit *in vitro* splicing and immunoprecipitate pre-messenger RNA and splicing products. Small nuclear RNA immunoprecipitation assays show that p255 is a component of the U5 small nuclear ribonucleoprotein (snRNP) and the [U4/U6.U5] tri-snRNP complex. In RNase protection assays, mAb CC3 immunoprecipitates fragments containing branch site and 3′ splice site sequences. As predicted for a [U4/U6.U5]-associated component, the recovery of the branch site-protected fragment requires binding of U2 snRNP and is inhibited by EDTA. p255 may correspond to the previously identified p220 protein, the mammalian analogue of the yeast PRP8 protein. Our results suggest that changes in the phosphorylation of p255 may be part of control mechanisms that interface splicing activity with nuclear organization.

INTRODUCTION

The removal of introns from nuclear pre-mRNAs takes place in the spliceosome which is assembled in a step-wise manner from small nuclear ribonucleoparticles (snRNP) and non-snRNP factors (reviewed in refs 1–3). Spliceosome formation is initiated by the binding of U1 snRNP to the 5′ splice site and U2AF binding to the polypyrimidine stretch near the 3′ splice site (4,5). Members of the SR family of proteins mediate an interaction between the U1 snRNP and U2AF, thereby committing splice site utilization (6,7). The binding of U2AF promotes the interaction of U2 snRNP with branch site sequences upstream of the polyprimidine stretch (8). Spliceosome formation continues through the association of the [U4/U6.U5] tri-snRNP complex (9). At this stage, both U5 snRNA and a protein of ~220 kDa interact with exon sequences immediately upstream of the 5′ splice site (10–12). Most likely, U1 snRNP binding to the 5′ splice site is destabilized at this point in the process. The spliceosome is then subjected to an important rearrangement in snRNP interactions: the U4 snRNA is released or destabilized, allowing U6 snRNA to engage in base-pairing interactions with U2 snRNA and with the 5′ splice site (reviewed in refs 3 and 13). Following cleavage at the 5′ splice site and concomittant branch formation, further changes in snRNP interactions occur in preparation for the second step of splicing. These include a change in the interaction between U6 and 5′ splice site sequences and an interaction between U5 and the first nucleotide of the second exon (12). Once cleavage at the 3′ splice junction and exon ligation take place, splicing factors dissociate, possibly to be used in the removal of other introns.

In the nucleus, snRNPs are concentrated in speckled structures but are also found uniformly distributed throughout the nucleoplasm (reviewed in ref. 14). SR proteins have also been localized to nuclear speckled regions (15,16). In contrast, U2AF does not localize to speckles but is diffusely distributed throughout the nucleoplasm (17). Nuclear speckles are made up of perichromatin fibrils which may represent nascent transcripts and interchromatin granule clusters which may constitute storage and/or assembly sites for splicing factors (18). When the cell enters mitosis, the speckled pattern breaks up while splicing factors are redistributed throughout the cytoplasm. Whereas the concentration of many splicing factors to speckles suggests that these subnuclear structures are sites of pre-mRNA processing, the localization of active spliceosomes in speckled areas is still controversial (for a review see ref. 19). A number of studies have indicated that RNA maturation can occur at sites of transcription which are not necessarily coincident with intranuclear speckles (20–22). Another source of considerable debate has been the relationship between subnuclear structures and the nuclear matrix, which is operationally defined as the insoluble structure that remains following the extraction of nuclei with non-ionic detergents, nucleases and high salt. The observation that several of the monoclonal antibodies raised against nuclear matrix components give speckled immunofluores-

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cence (23–25) suggests that snRNPs, SR proteins and possibly spliceosomes are associated with the nuclear matrix.

Recent work suggests that the phosphorylation of splicing factors represents an important mechanism that intimately couples splicing activity with changes in the nuclear architecture during the cell cycle. Splicing factors containing serine- and arginine-rich domains (e.g. SR proteins and U2AF^{65}) are phosphorylated by SRPK1, a kinase that promotes the disassembly of nuclear speckles (26). The expression of SRPK1 is upregulated at mitosis and high concentration of SRPK1 can block *in vitro* splicing (26). In contrast, dephosphorylation of the U1 snRNP 70 kDa protein, which also contains serine- and arginine-rich domains, is essential for splicing activity (27). The serine/threonine phosphatase PP1 can dephosphorylate SR proteins *in vitro* and prevents spliceosome assembly (28), while phosphatase inhibitors block splicing but not complex formation (28,29). Thus, the reversible phosphorylation of splicing factors is likely to play an important role in modulating splicing activity and the organization of splicing factors in the nucleus.

We have characterized a monoclonal antibody that recognizes a phosphorylated epitope present on a 255 kDa protein (25,30). We demonstrate that p255 is an integral component of the [U4/U6.U5] tri-snRNP complex. The antibody can inhibit *in vitro* splicing and immunoprecipitation assays indicating that p255 associates with the spliceosome following U2 snRNP binding. The tight association of p255 with the nuclear matrix (25) and the cell cycle-dependent phosphorylation of p255 (25,30) suggest that p255 may be involved in linking splicing activity with cell cycle-dependent modifications of the nuclear structure.

MATERIALS AND METHODS

Antibody preparation, immunofluorescence and Western blotting

MAb CC3 was obtained following immunization of a Balb/C mouse with pharyngeal regions isolated from 72 h chick embryos (30). The culture supernatant of CC3 hybridoma was collected and used direcly in immunofluorescence assays and immunoblots (30). For the immunoprecipitation assays, mAb CC3 was purified by affinity chromatography on a protein A–Sepharose column. Immunoblotting was performed as described in Bisotto *et al.* (25) and Thibodeau and Vincent (30)

Transcripts, nuclear extract preparation and *in vitro* splicing

Plasmids pSP64-HβΔ6, -β^{110} and pSPAd have been described previously (31,32). pSP64-HβΔ6 and -β^{110} were cut either with *Bam*HI or *Fnu*4H and pSPAd was cut with *Hin*cII and transcribed with SP6 RNA polymerase in 25 µl mixture containing 5 µl of ^{32}P-UTP as described (33).

HeLa cell nuclear and S-100 extracts used for splicing were prepared as described in Dignam *et al.* (34). The reactions were set up according to Krainer *et al.* (35) for the times indicated. ATP-depleted extracts were prepared according to Michaud and Reed (36). Targeted degradation of U2 snRNA in nuclear extracts was accomplished with oligonucleotide and RNase H as described previously (37). Aliquots of splicing reactions were taken at various times and the RNA was isolated and resolved on 10% acrylamide/7 M urea gels.

Antibody inhibitions and immunoprecipitations

In addition to mAb CC3, we used the monoclonal anti-Sm (Y12) antibodies, patient autoantibodies against U2 snRNP (Ya, kindly provided by J. A. Steitz, Yale University), mAb GP39 (raised against recombinant CD-40 ligand gp39) and mAb F51H2 (raised against IFAPα-400, a developmentally regulated cytoskeletal protein of the chick embryo). Immunoprecipitation/protection assays on the β-globin substrate were performed in 25 µl splicing reactions as described (31,37,38). Briefly, antibodies and 5 µl of RNase T1 (Calbiochem; 29 U/µl) were added to splicing mixtures which were kept on ice for 30 min. A 4 mg amount of protein A–Sepharose {in 50 µl of NET-2 [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% (v/v) NP-40 and 0.5 mM DTT]} was added to each sample and incubation was continued for 15 min. The immunoprecipitate was then washed 5× with NET-2 buffer or with a similar buffer containing the salt concentration specified in the text. Immunoprecipitation of snRNAs, splicing substrate, intermediates and products was accomplished exactly as above except that RNase T1 was omitted. HeLa snRNAs were 3′ end labeled by using [^{32}P]pCp (Amersham) and T4 RNA ligase (Promega) as described in England *et al.* (39).

Glycerol gradient sedimentation

HeLa nuclear extract (1 ml) dialyzed in buffer G (20 mM HEPES pH 7.9, 250 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 20 mM β-glycerophosphate, 5% glycerol) was loaded on a 11 ml 10–30% (v/v) glycerol gradient prepared with buffer G. The gradient was centrifuged in a Beckman SW 40 Ti rotor at 29 000 r.p.m. for 18 h as described in Behrens and Lührmann (40). Twenty-three 0.5 ml fractions were collected from bottom to top. The RNA content from 100 µl of each fraction was purified by phenol extraction and resolved on a 10% acrylamide/7 M urea gel. Another 100 µl aliquot was immunoprecipitated with 5 µl of mAb CC3 as described above.

RESULTS

MAb CC3 recognizes a nuclear phosphoprotein of 255 kDa

The monoclonal antibody CC3 was selected from a library of monoclonal antibodies raised against chick embryo branchial arches in a search for developmental markers (41). In interphase cells, mAb CC3 reacts with a phylogenetically well-conserved 255 kDa protein localized to the nucleus (Fig. 1A, lane 1) (30). Immunostaining reveals a typical speckled distribution (25,30) characteristic of the staining obtained with antibodies against SR proteins, snRNP antigens and other splicing factors (14,16,26). At mitosis, mAb CC3 shows a diffuse pattern of reactivity but preferentially associates with the mitotic apparatus (spindle and centrosomes) (30,41). Western analysis reveals a completely new set of proteins whose migration is distinct from p255 (Fig. 1A, lane 2) (30). It is unclear whether some of these proteins represent phosphorylated isoforms of p255 or, alternatively, whether they represent different mitosis-specific proteins sharing with p255 a common phosphorylated epitope. The reactivity of mAb CC3 depended on the phosphorylation of the antigens since it was abolished by phosphatase treatment (30). Immunoblot assays showed that p255 was present in a HeLa nuclear extract and was detectable in a HeLa post-nuclear S-100 supernatant after longer exposures (Fig. 1B, lanes 1–2). Western blot analyses also showed that mAb CC3 did not react with a mixture of purified calf

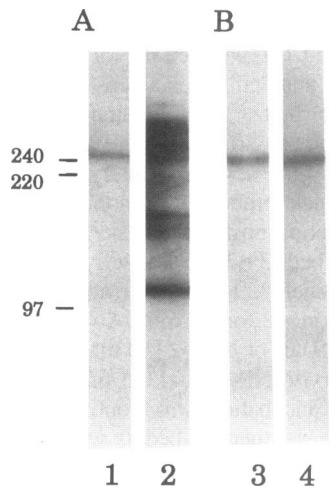

Figure 1. Immunoreactivity of mAb CC3 on different HeLa cell extracts. (**A**) Immunoblot analysis of total extracts from unsynchronized (lane 1; >85% interphase cells) and colcemid-arrested mitotic cells (lane 2) separated on 5–15% polyacrylamide gradient gel (5). Both wells were loaded with the equivalent of 1.5×10^5 cells. (**B**) Immunoblot analysis of HeLa cell nuclear (lane 1) and S-100 extracts (lane 2). 150 µg of total proteins were loaded in each well. Proteins were transfered onto nitrocellulose and probed with mAb CC3 as described (58). Note that lane 2 (S-100) was exposed 4-fold longer than lane 1 (nuclear extract). Molecular weigths of the α (240) and β spectrin (220) subunits and phosphorylase B (97) used as M_r markers are given in kDa.

thymus SR proteins, while the same preparation was efficiently recognized by mAb 104 (data not shown), an antibody specific for the phosphorylated epitope of SR proteins (42).

A recent study has shown that p255 is resistant to extraction with non-ionic detergents, nucleases and high salt and may thus be defined biochemically as a nuclear matrix component (25). Double labelling immunofluorescence studies revealed that p255 and SC35 display identical spacio-temporal localization (16,25,39). Thus, mAb CC3 recognizes a phosphorylated epitope on the 255 kDa interphase protein which is a component of the nuclear matrix.

MAb CC3 can inhibit *in vitro* splicing

The nuclear distribution of p255 suggests that it could be involved in splicing. To test this possibility we asked whether mAb CC3 could inhibit the *in vitro* splicing of the adenovirus major late and human β-globin pre-mRNAs. Addition of increasing amounts of mAb CC3 to splicing mixtures resulted in the inhibition of adenovirus splicing (Fig. 2A, lanes 2–3). The appearance of both splicing intermediates and products was compromised by the addition of mAb CC3 (10-fold inhibition). A decrease in the production of spliced mRNA could not be observed because mRNA was not detected in the control reaction (lane 1), possibly because it was degraded. Addition of equivalent amounts of an irrelevant control antibody (mAb GP39) did not interfere with splicing (Fig. 2A, lanes 4–5). β-globin splicing was less sensitive than adenovirus splicing to the presence of mAb CC3 (Fig. 2B, lanes 2–3; ~2-fold inhibition). Addition of mAb GP39 had no effect (Fig. 2B, lanes 4 and 5). These results suggest that p255 associates with splicing complexes. However, the finding that splicing of the adenovirus and β-globin pre-mRNAs are differentially affected by mAb CC3 suggests that the interaction of p255 with different pre-mRNAs is not identical.

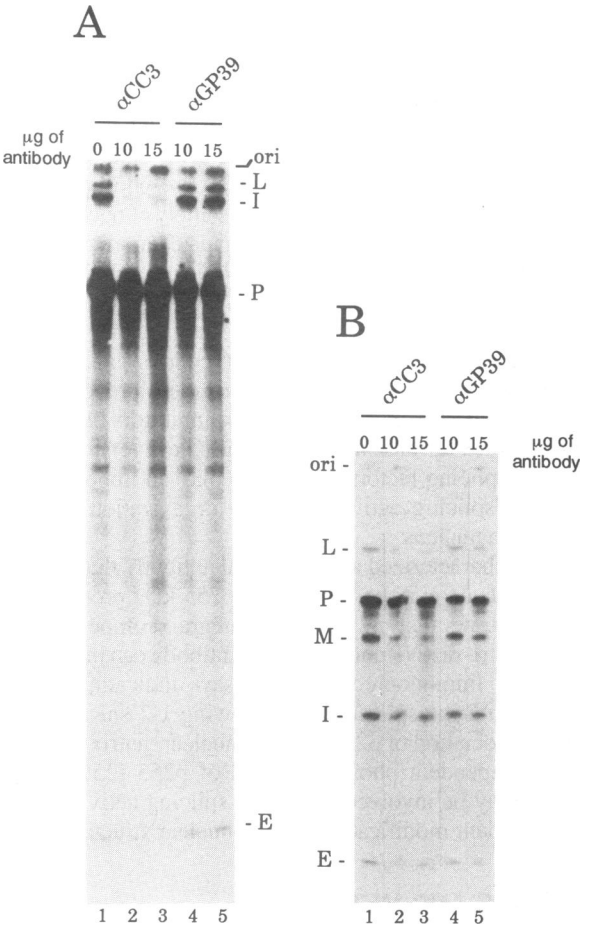

Figure 2. MAb CC3 inhibits *in vitro* splicing. The indicated amounts of mAb CC3 or an unrelated monoclonal antibody (mAb GP39) were added to HeLa splicing mixtures (0.5 µl of nuclear extract in a total of 12.5 µl) lacking pre-mRNA substrates. Following a 30 min incubation on ice, the ^{32}P-labeled adenovirus major late (panel A, Ad/*Hin*cII) or human β-globin pre-mRNA (panel B, HβΔ6/*Bam*HI) was added and the mixtures were incubated for 2 h at 30°C. Following RNA extraction, labeled RNA molecules were separated on a 10% polyacrylamide/7 M urea gel. The origin of the gel (ori) and the position of the pre-mRNA (P), lariat intermediate (L), lariat intron (I), excised 5′ exon (E) and mRNA (M) are indicated.

p255 associates with splicing complexes

To confirm that p255 associates with splicing complexes we performed an immunoprecipitation assay with mAb CC3 at various times during a splicing reaction. MAb CC3 was added directly to complete splicing mixtures containing a labeled human β-globin pre-mRNA (HβΔ6/B). RNA species were recovered by binding to protein A–Sepharose and analyzed by gel electrophoresis. At time zero, ~2% of the input globin substrate was immunoprecipitated from the splicing reaction (Fig. 3, lane 10). As the reaction proceeded, the recovery of the precursor RNA declined but splicing intermediates, excised intron and mRNA were detected in immunoprecipitates (Fig. 3, lanes 11–12). Similar results were obtained with a related globin pre-mRNA containing a shortened second exon that prevents the second step of splicing (HβΔ6/F; Fig. 3, lanes 4–9). RNA

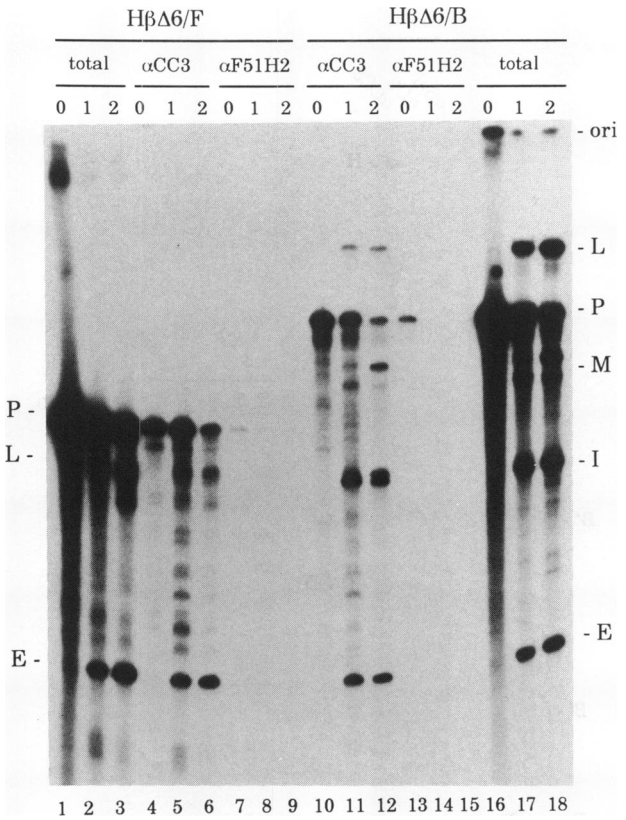

Figure 3. MAb CC3 immunoprecipitates splicing products. HeLa splicing mixtures containing the human β-globin pre-mRNA (HβΔ6/*Bam*HI; lanes 10–18) or a shortened derivative (HβΔ6/Fnu4H; lanes 1–9) were incubated for 0, 1 or 2 h at 30°C. Immunoprecipitation assays were then performed with either 3 µl of mAb CC3 (lanes 4–6 and 10–12) or 3 µl of an unrelated antibody (mAb F51H2; lanes 7–9 and 13–15). An aliquot representing 1/10th of each splicing mixture was also directly processed at the indicated times (total). Labeled RNAs were then resolved on a denaturing gel. The origin of the gel and the position of the pre-mRNA (P), lariat intermediate (L), lariat intron (I), excised 5′ exon (E) and mRNA (M) are indicated.

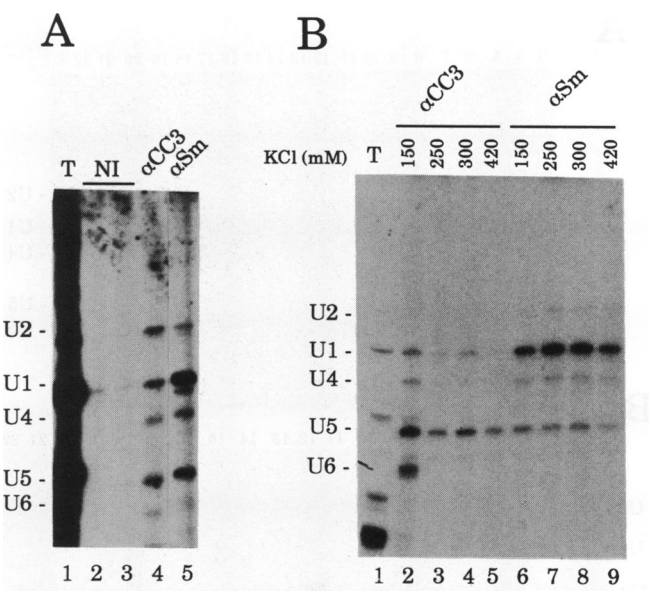

Figure 4. p255 interacts with snRNAs. (**A**) Splicing mixtures containing HeLa nuclear extracts, ATP and magnesium but lacking exogenously added pre-mRNA were incubated 15 min at 30°C and immunoprecipitated with either non-immune sera (NI; lanes 2 and 3), mAb CC3 (α-CC3; lane 4) or the Y12 antibody (α-Sm; lane 5). The immunoprecipitated RNAs were extracted, labeled with [^{32}P]pCp and resolved on a 10% polyacrylamide/7 M urea gel. (**B**) HeLa splicing mixtures incubated in the absence of magnesium were used in immunoprecipitation assays with mAb CC3 (α-CC3; lanes 1–5) or the Y12 antibody (α-Sm; lanes 6–9). The immunoprecipitates were washed with a buffer containing the indicated concentration of KCl. An aliquot of the total splicing mixture or extract was used in each panel (T). The position of each snRNA is indicated in each panel.

molecules were not significantly immunoprecipitated when the experiment was carried out with an irrelevant monoclonal antibody (mAb F51H2; Fig. 3, lanes 7–9 and 13–15) or when anti-p255 immunoprecipitation was performed with a β-globin mRNA (data not shown). Thus, the profile of immunoprecipitation obtained with mAb CC3 is diagnostic of an association of p255 with the spliceosome and suggests that p255 interacts with splicing complexes during both steps of splicing.

p255 is a component of the U5 snRNP and the [U4/U6.U5] tri-snRNP

To investigate whether p255 is a snRNP component, snRNAs were immunoprecipitated from a nuclear extract with mAb CC3. The precipitated RNAs were then 3′ end labeled with [^{32}P]pCp and separated on a polyacrylamide gel. The anti-Sm antibody immunoprecipitated all the major snRNAs except U6 (Fig. 4A, lane 5). U6 snRNA is normally underrepresented in this assay because the majority of U6 molecules contain a cyclic phosphate at the 3′ end that prevents efficient pCp labeling (43). MAb CC3 immunoprecipitated all the major snRNAs including U6 (Fig. 4A, lane 4). Compared with the anti-Sm immunoprecipitate, U6 snRNA was overrepresented while U1 was underrepresented. For unclear reasons, the recovery of U2 snRNA by mAb CC3 was lost in splicing mixtures lacking magnesium (Fig. 4B, lane 2). When immunoprecipitates were washed at higher salt concentrations, the recovery of snRNAs with the anti-Sm antibody was not affected (Fig. 4B, lanes 6–9). In contrast, the recovery of U1, U4 and U6 by mAb CC3 decreased at higher salt concentrations; U5 being the only snRNA efficiently recovered at 420 mM KCl (Fig. 4B, lanes 2–5). These results suggest that p255 is a stable component of the U5 snRNP.

U6 RNA molecules with additional U residues and lacking a cyclic phosphate at their 3′ end are recruited for the assembly of U4/U6, [U4/U6.U5] snRNP complexes and spliceosomes (44–46). Thus, the recovery of U6 snRNA molecules that can be 3′ end-labeled indicates the absence of a cyclic phosphate at this extremity and suggests that p255 may also be a component of the [U4/U6.U5] tri-snRNP. To confirm the presence of p255 in the [U4/U6.U5] tri-snRNP, we fractionated a HeLa nuclear extract by glycerol gradient centrifugation in 250 mM KCl. In these conditions, the U4/U6 and U5 snRNPs sediment quantitatively together in the form of the 25S [U4/U6.U5] tri-snRNP complex (47). We observed a fractionation profile of the U4, U5 and U6 snRNAs consistent with the formation of [U4/U6.U5] tri-snRNP (Fig. 5A, fractions 6–11). An aliquot of each fraction was immunoprecipitated with mAb CC3. The snRNAs recovered were labeled with [^{32}P]pCp and separated on a gel (Fig. 5B). The

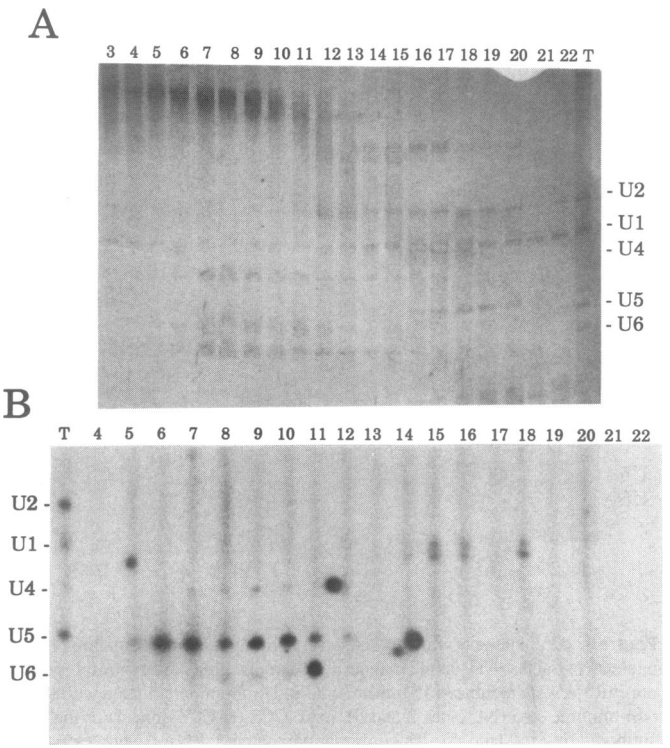

Figure 5. p255 is a component of the [U4/U6.U5] tri-snRNP complex. (**A**) A HeLa nuclear extract adjusted to 250 mM KCl was fractionated on a 10–30% glycerol gradient. The RNA species presents in each fraction were extracted and resolved on a denaturing gel which was silver-stained. (**B**) An aliquot of each fraction was immunoprecipitated with mAb CC3 and the RNA species were 3′ end-labeled with [^{32}P]pCp. Gel-fractionation of the snRNAs was performed as above. The position of each snRNA is indicated.

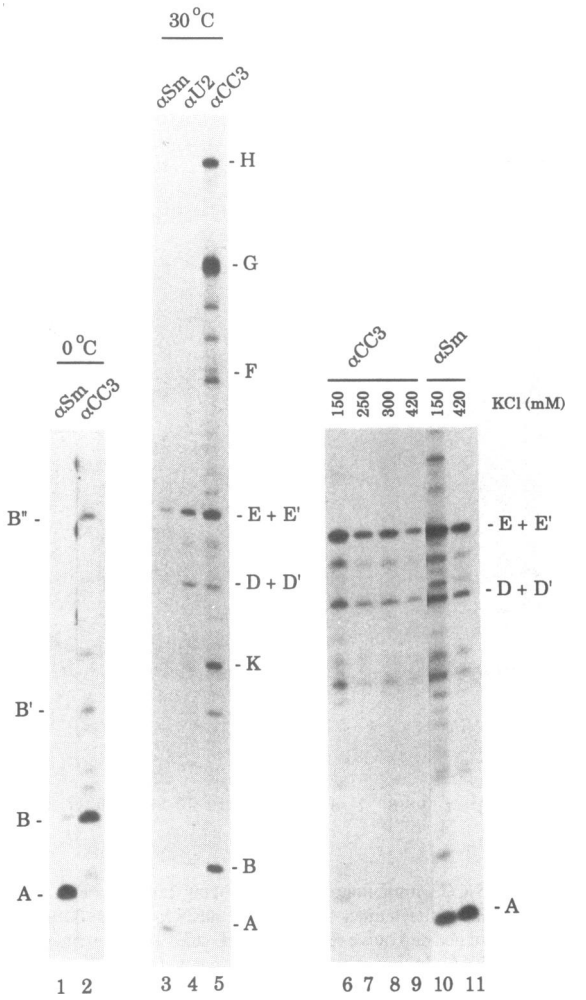

Figure 6. Interaction of p255 with the globin pre-mRNA. HeLa splicing mixtures containing the HβΔ6/Fnu4H RNA were incubated at 0°C (lanes 1 and 2) or at 30°C for 45 min (lanes 3–11). RNAse T1 protection/immunoprecipitation assays were then performed on ice using either 10 µl of the monoclonal antibody Y12 (α-Sm; lanes 1, 3, 10 and 11), 10 µl of anti-U2(RNP) antibody (α-U2; lane 4) or 3 µl of mAb CC3 (α-CC3; lanes 2 and 5–9). The immunoprecipitates were washed with a buffer containing 150 mM NaCl (lanes 1–5) or a buffer containing KCl at the concentration indicated (lanes 6–11). Labeled protected fragments were separated on a 15% polyacrylamide/7 M urea gel. The size of the protected fragments and their respective position in the pre-mRNA are indicated in the text and in Chabot and Steitz (31). Note that fragments K, F, G and H were clearly visible in an overexposure of lanes 3 and 4 (not shown).

results indicate that mAb CC3 immunoprecipitates U4, U5 and U6 snRNAs in fractions where these snRNAs form a tri-snRNP complex, suggesting that p255 is a component of the [U4/U6.U5] snRNP particle.

p255 can interact with components bound to the 3′ splice site

To determine whether p255 interacts with specific regions of the pre-mRNA, we performed an RNase T1 protection/immunoprecipitation assay using the β-globin substrate (31,37,38). This assay consists in adding ribonuclease T1 along with the antibody to splicing mixtures. The protected RNA fragments are recovered with protein A–Sepharose and analyzed by gel electrophoresis. As a control, we performed this experiment with an anti-Sm monoclonal antibody (Y12). Using extracts kept at 0°C, the anti-Sm antibody immunoprecipates a fragment 15 nucleotides (nt) in length that corresponds to the 5′ splice site bound by the U1 snRNP (37) (fragment A; Fig. 6, lane 1). In addition, a 19 nt fragment (fragment B) mapping at the 3′ splice site is recovered with reduced efficiency. Previous work has indicated that fragment B is immunoprecipitated by virtue of its association with the U5 snRNP (38). A U5-specific protein of 100 kDa (IBP), reacting with anti-Sm antibodies, was later shown to recognize the polypyrimidine-rich 3′ end of introns (48). A polypeptide of 70 kDa that reacts with anti-Sm antibodies and recognizes the same 3′ splice site sequence was also described as a possible U5-specific protein (49). This 70 kDa protein likely represents a proteolytic product of IBP (see ref. 50).

In extracts incubated at 0°C, mAb CC3 antibody selectively immunoprecipitated fragment B (Fig. 6, lane 2). Larger fragments (B′ and B″) corresponding to more extensive upstream intron protection (38) were also recovered. Given that p255 is a component of U5 snRNP, our result suggests that the recovery of 3′ splice site sequences by mAb CC3 occurs via U5 snRNP binding to the 3′ splice site.

MAb CC3 immunoprecipitates branch site fragments in a U2 snRNP-dependent manner

RNase T1 protection/immunoprecipitation experiments were next performed following incubation of splicing mixtures at 30°C. In these conditions, anti-Sm and anti-U2(RNP) antibodies immunoprecipitate a similar set of protected fragments derived from assembled spliceosomes (31) (Fig. 6, lanes 3 and 4). Fragments E and D correspond to U2 snRNP binding to branch site sequences of the β-globin pre-mRNA while E′ and D′ correspond to an extended protection of U1 snRNP binding to the 5′ splice site. Fragment F contains both branch site and 3′ splice site sequences. Fragments G and H contain branch site and 3′ splice site sequences derived from lariat molecules while fragment K maps to the central portion of the free 5′ exon. MAb CC3 immunoprecipitated the same set of protected fragments (Fig. 6, lane 5). However, relative to protected fragments E + E′, fragments K, F, G and H were recovered considerably more efficiently with mAb CC3 than with the anti-Sm and anti-U2(RNP) antibodies. The interaction of p255 with the pre-mRNA substrate was very stable since the recovery of fragments E + E′ resisted washes performed in buffers containing up to 420 mM KCl (Fig. 6, lanes 6–9).

Since U2 snRNP binding is required for the entry of the [U4/U6.U5] tri-snRNP complex into spliceosomes, the recovery of branch site-protected fragments with mAb CC3 should require U2 snRNP binding. We tested this prediction by performing protection/immunoprecipitation assays in extracts in which the 5′ end of U2 snRNA was targeted for degradation by deoxyoligonucleotides and RNase H. SnRNA degradation was verified by pCp labeling (data not shown). In an extract depleted of U2 snRNP in this fashion, the recovery of fragments E + E′ by anti-Sm antibody was reduced considerably (Fig. 7A, lane 1). For unclear reasons, immunoprecipitation with mAb CC3 yielded a complex mixture of protected fragments (Fig. 7A, lane 4). However, protected fragments E + E′ were absent from this mixture. This result suggests that a functional U2 snRNP is required for the association of p255 with splicing complexes. This conclusion was confirmed by a protection assay performed in an extract depleted of ATP, which allows formation of commitment complexes containing U1 snRNP, U2AF and SR proteins but prevents the assembly of U2 snRNP-containing complexes (5,7,36). In ATP-depleted extracts, both anti-Sm and mAb CC3 antibodies failed to recover protected fragments diagnostic of U2 snRNP and p255 binding (Fig. 7A, lanes 3 and 6). We could not accomplish protection assays in extracts targeted for the degradation of U1 or U4 snRNAs since treatments resulted in only a partial degradation of the targeted snRNAs (data not shown).

To ascertain the composition of the E + E′ band obtained with mAb CC3, we used the mutant β-globin transcript HβΔ6-β^{110}. This mutant is spliced exclusively at an AG dinucleotide created by a G-to-A transition 21 nt upstream of the 3′ splice junction (51). The mutation changes the length of the branch site-protected fragment from 42 to 43 nt and therefore allows the assessment of the relative proportion of E and E′ (31). The result of the protection assay with HβΔ6-β^{110} indicates that mAb CC3 immunoprecipitated mostly branch site sequences (Fig. 7B, lane 3). To address the association of p255 with events occurring after U2 snRNP binding, we used extracts containing 2.5 mM EDTA. The presence of EDTA allows U2 snRNP binding but inefficient or aberrant binding of the [U4/U6.U5] tri-snRNP (11). Thus, EDTA treatment allows us to test whether incorporation of the

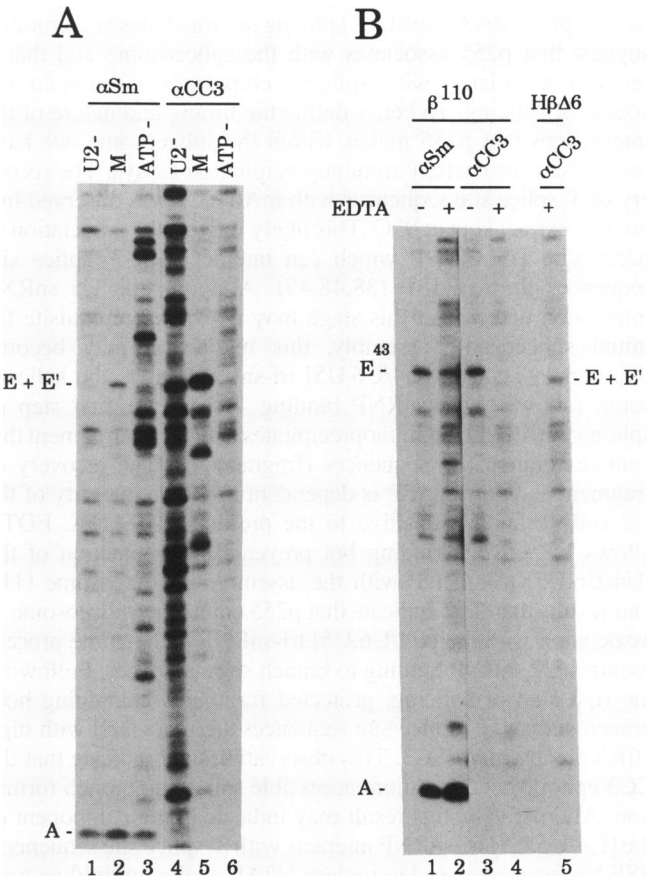

Figure 7. Requirements for the interaction of p255 with the pre-mRNA. (**A**) Nuclear extracts depleted of U2 snRNP by oligonucleotide-targeted degradation (lanes 1 and 4), mock-incubated (lanes 2 and 5) or depleted of ATP (lanes 3 and 6) were incubated with HβΔ6/Fnu4H RNA for 45 min at 30°C. RNAse T1 protection/immunoprecipitation assays using the monoclonal antibody Y12 (α-Sm; lanes 1–3) or CC3 (α-CC3; lanes 4–6) were then performed as in Figure 4. (**B**) RNase T1 protection/immunoprecipitation assays were carried out with extracts incubated with either HβΔβ^{110}/Fnu4H (β^{110}; lanes 1–4) or HβΔ6/Fnu4H (lane 5) in the absence (lanes 1 and 3) or the presence (lanes 2, 4 and 5) of 2.5 mM EDTA. The antibodies used were the same as in panel A. The position of E + E′ (42 nt), E^{43} (43 nt) and A (15 nt) is indicated.

[U4/U6.U5] tri-snRNP in the spliceosome is required for the recovery of branch site-protected fragments with mAb CC3. Using HβΔ6-β^{110} as a substrate, we observed that the presence of EDTA abrogated the recovery of branch site-protected fragments with mAb CC3 (Fig. 7B, lane 4). In contrast, anti-Sm antibody continued to efficiently immunoprecipitate branch site fragments protected by U2 snRNP in the presence of EDTA (Fig. 7B, lane 2). These results suggest that the entry of the [U4/U6.U5] tri-snRNP in the spliceosome is required for the association of p255 with splicing complexes.

DISCUSSION

p255 as a spliceosome component

Immunoprecipitation assays with mAb CC3 indicate that p255 stably associates with U5 snRNA. Moreover, gradient fractionation indicates that p255 is a component of the [U4/U6.U5]

tri-snRNP complex. The ability of mAb CC3 to immunoprecipitate the pre-mRNA substrate, splicing intermediates and products suggest that p255 associates with the spliceosome and that it remains associated with splicing complexes throughout the course of splicing. To better define the timing and nature of the interactions that p255 makes within the spliceosome, we have used RNase protection/immunoprecipitation assays. The recovery of 3′ splice site sequences with mAb CC3 was observed in a nuclear extract kept at 0°C. This likely reflects the association of p255 with U5 snRNP which can interact with 3′ splice site sequences through IBP (38,48,49). Although the U5 snRNP interaction detected at this stage may not be a prerequisite for initial spliceosome assembly, this interaction may become important when the [U4/U6.U5] tri-snRNP enters the spliceosome following U2 snRNP binding. Before the first step of splicing, mAb CC3 immunoprecipitates a protected fragment that contains branch site sequences (fragment E). The recovery of fragment E requires ATP, is dependent upon the integrity of the U2 snRNP and is sensitive to the presence of EDTA. EDTA allows U2 snRNP binding but prevents the interaction of the [U4/U6.U5] tri-snRNP with the assembling spliceosome (11). Our results therefore indicate that p255 enters the spliceosome in association with the [U4/U6.U5] tri-snRNP and that the process requires U2 snRNP binding to branch site sequences. Following the first step of splicing, protected fragments containing both branch site and 3′ splice site sequences are recovered with high efficiency by mAb CC3. This observation may indicate that the CC3 epitope becomes more accessible following branch formation. Alternatively, this result may indicate that a component of the [U4/U6.U5] tri-snRNP interacts with 3′ splice site sequences. PSF has been proposed to replace U2AF on the pyrimidine tract in preparation for the first step of splicing (52). PSF may be identical to the U5 snRNP protein IBP because PSF also binds to the polypyrimidine track (53) and, like IBP, is a protein of 100 kDa that produces a 68 kDa breakdown product (52). Thus, the binding of IBP/PSF to 3′ splice site sequences may play an important role in positioning the [U4/U6.U5] tri-snRNP in the assembling spliceosome and may lead to the release of U2AF.

The immunoprecipitation of the same protected fragments by mAb CC3 and the anti-U2(RNP) antibody may indicate that p255 interacts with U2 snRNP in the spliceosome. Following the entry of the [U4/U6.U5] tri-snRNP in the spliceosome, base pairing interactions occur between U2 and U6 snRNAs (54–56). Thus, the association of p255 with the U2/U6.U5 complex is likely to be responsible for the recovery of identical protected fragments by mAb CC3 and the anti-U2(RNP) antibody. It is also possible that p255 interacts directly with nucleotides at or near the branch site.

Relationship between p255, p220 and PRP8

Our results raise the possibility that p255 is identical to p220, the mammalian analogue of the yeast PRP8 protein and the largest identified protein in the spliceosome. Based on cross-reactivity with an anti-PRP8 antibody, p220 has been detected as a protein of >200 kDa often appearing as a doublet in HeLa extracts, in purified U5 snRNP and in [U4/U6.U5] particles (40,50,57,58). p220 cross-links to pre-mRNAs and to exon sequences adjacent to the 5′ splice site in HeLa extracts incubated under splicing conditions (10,59). Recently, a 220 kDa spliceosomal protein was found to cross-link to a branch region photo-probe (60). The functional analogy between PRP8 and p255 is further reinforced by the recent demonstration that PRP8-specific antibodies also immunoprecipitate protected fragments containing branch site and 3′ splice site sequences derived from lariat molecules (61). Although our results suggest that p220 and p255 represent the same protein, we have remained unable to detect cross-reactivity between an anti-PRP8 antibody and any high-molecular weight HeLa protein following immunoprecipitation with either an anti-Sm or mAb CC3. Consequently, it remains possible that p255 is distinct from p220 and further work will be required to clarify this issue.

p255 phosphorylation, nuclear organization and splicing

Recent results indicate that p255 is a component of the nuclear matrix (25). p255 colocalizes with the splicing factor SC35 at different stages of the cell cycle and their co-distribution is not disrupted by heat shock or treatment with DNase I and RNases (5,51). A number of other nuclear matrix proteins have been reported through the use of monoclonal antibodies (23,24,62–68). Among them, the 240 kDa phosphoprotein cytostellin co-localizes with SC35 but is not detected in crude or purified spliceosomes and appears to be part of distinct molecular complexes (24). The nuclear matrix protein NuMA (230 kDa) localizes with splicing factors in interphase nuclei and associates with pre-mRNA in nuclear extracts incubated under splicing conditions (69). The role of these proteins in splicing and their relationship to p255 remains to be clarified. Recently, the nuclear matrix antigens B1C8 (180 kDa), H1B2 (240 kDa) and B4A11 (~300 kDa) were shown to co-localize with SC35 and to associate with exon-containing splicing complexes (68). In contrast to p255 however, B1C8, H1B2 and B4A11 are not stable components of snRNPs and appear to be related to members of the SR family of proteins (68). The involvement of the nuclear matrix in RNA processing events is supported by the demonstration that the addition of nuclear extracts to matrix-associated pre-mRNAs leads to splicing without a temporal lag in the reaction (70). Moreover, antibodies against some matrix proteins inhibit *in vitro* splicing (64,68, our results). Formally however, these results do not prove that spliceosomes interact with the nuclear matrix. It remains possible that splicing factors are stored in nuclear speckles only to be recruited by spliceosomes assembling at the site of transcription or during RNA transport.

A relationship between the phosphorylation of splicing factors and nuclear organization has recently been revealed by the observation that the phosphorylation of SR proteins increases at mitosis (26). SR proteins are phosphorylated by SRPK1, a cell cycle-regulated kinase which inhibits *in vitro* splicing in a concentration-dependent manner and which promotes the disassembly of nuclear speckles (26,71). While it is not yet known whether p255 is a substrate for SRPK1, the phosphorylation of p255 also changes dramatically at mitosis: mAb CC3 reveals a new set of M phase-specific proteins. Experiments are underway to establish the relationship, if any, between p255 and the M phase-specific proteins. Like SC35, proteins carrying the CC3 epitope diffuse into the cytoplasm during metaphase but reorganize into cytoplasmic speckles during the anaphase–telophase transition (25,30). Thus, changes in the level of phosphorylation of p255 may regulate its activity and/or modulate its intranuclear localization and hence its availability. Reversible phosphorylation of p255 may be intimately coupled with cell cycle decisions and could provide a mechanism to attune spliceosome assembly

with changes in nuclear organization. Interestingly, recent genetic studies in the budding yeast have uncovered a role for PRP8 in cell cycle progression (72). This result strengthens the functional analogy between PRP8 and p255, and suggests a crucial role for these proteins in the coordination of biological pathways.

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Note added in proof

Recent experiments suggest that p255 is distinct from p220. Antibodies against p220 (kindly provided by Dr Melissa Moore) recognise a protein that migrates more rapidly than p255. Consistent with our observations however, mAb CC3 can immunoprecipitate p220 from a nuclear extract indicating that at least a fraction of p225 co-exists in the same complex as p220.

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