Phosphorylated Ser/Arg-rich proteins: Limiting factors in the assembly of 200S large nuclear ribonucleoprotein particles

(pre-mRNA splicing factors/sucrose gradients/Western blotting/immunoprecipitation)

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ABSTRACT We have previously shown that specific nuclear pre-mRNA transcripts and their splicing products, as well as the general population of nuclear poly(A)⁺ RNA, are packaged in large nuclear ribonucleoprotein (hnRNP) particles that sediment at the 200S region in sucrose gradients. The hnRNP particles contain all uridine-rich small nuclear ribonucleoprotein complexes required for pre-mRNA splicing, as well as protein splicing factors. In this paper we show that all of the phosphorylated, mAb 104 detectable, Ser/Arg-rich essential splicing factors (SR proteins) in the nucleoplasm are integral components of the hnRNP particles, whereas only part of the essential splicing factor U2AF⁶⁵ (U2 snRNP auxiliary factor) and the polypyrimidine tract binding protein (PTB) are associated with these particles. This finding suggests a limiting role for SR proteins in the assembly of the hnRNP particles. We further show that the structural integrity of hnRNP particles is sensitive to variations in the phosphorylation levels of the SR proteins.

Numerous studies of splicing *in vitro*, using cell-free nuclear extracts and pre-mRNA-like synthetic molecules, established that the first chemical step of this transformation must be preceded by the gradual assembly of a multi-component ribonucleoprotein (RNP) complex called the spliceosome. These studies defined the minimal spliceosomal components, which include the U1, U2, U5, and U4/U6 small nuclear RNPs (snRNPs) and a large number of proteins that are not associated with the uridine-rich (U) snRNPs (for reviews see refs. 1–3). Among the non-snRNP spliceosomal proteins that have been distinguished as essential splicing factors are: U2AF (U2 snRNP auxiliary factor), which binds to the polypyrimidine tract near the 3' splice site and is required for the binding of U2 snRNP to the branch site (4–6); and the Ser/Arg-rich proteins (SR proteins), a family of conserved proteins whose carboxyl-terminal domains are rich in serine and arginine residues (7–10). Many animal cells express a set of at least six SR proteins of similar molecular weights that range between 20 and 75 kDa, all of which share a phosphorylated epitope recognized by monoclonal antibody mAb 104 (8, 11): SRp20, SRp30a,b, SRp40, SRp55, and SRp75. SRp30a and SRp30b have been identified as the essential splicing factors ASF/SF2 (12, 13) and PR264/SC35 (14, 15), respectively.

SR proteins were proposed to play multiple roles in spliceosome assembly. They were shown to function in 5' splice-site selection by assisting the binding of U1 snRNP to the pre-mRNA (16–18), thereby committing the pre-mRNA to the splicing pathway (19). Furthermore, excess of SR proteins was shown to circumvent the requirement for U1 snRNP for splicing (20, 21). SR proteins were also shown to collaborate with other proteins, such as U2AF, in 3' splice-site selection. In doing so, SR proteins were proposed to bridge between U1

and U2 snRNPs bound to their cognate sites on the pre-mRNA, thus mediating interaction between the 5' and 3' splice sites (22, 23). Recent studies have shown that the SR proteins are also required for the binding of U4/U6.U5 tri-snRNP complex to the spliceosome (24). The distinct functions in alternative splicing, which have been assigned to individual members of the SR protein family (25, 26), may thus be explained by their participation in early stages of spliceosome assembly. The various activities of SR proteins involve interactions with other proteins and with RNA. For example, the interaction of SF2/ASF (17), as well as that of SC35 (23), with U1 snRNP is mediated by direct protein–protein interactions with the U1 specific 70K protein, and possibly with other proteins that contain the SR domain. Direct protein–RNA interactions between SR proteins and pre-mRNA have been shown to occur within a set of complexes related to the earliest stages of the spliceosome assembly *in vitro* (27). Because the SR proteins are distinguished by their phosphorylated SR domain, their involvement in these interactions is most likely regulated by the phosphorylation state of their SR domain (28, 29), as well as by the phosphorylation state of other spliceosomal proteins containing an SR domain such as the U1 specific 70K protein (30).

In view of the proposed role of the SR proteins in pre-mRNA splicing, this family of proteins is expected to be associated with the RNA processing machinery *in vivo*. Pre-mRNA splicing *in vivo* apparently occurs on structures more complex than individual spliceosomes. This should not be surprising, because the splicing machinery of a living cell is expected to package and process multi-intronic pre-mRNAs, rather than the mono-intronic pre-mRNAs, which are typically used to assemble spliceosomes *in vitro*. Indeed, we have shown that several specific nuclear pre-mRNAs and their splicing products, as well as the general population of nuclear poly(A)⁺ RNA, are packaged in compact large nuclear RNP (hnRNP) particles that invariably sediment at the 200S region in sucrose gradients (31–34). The hnRNP particles contain all U snRNPs required for splicing (ref. 35 and this paper), as well as hnRNP proteins and non-snRNP splicing factors (ref. 36 and this paper). These and other studies have led us to propose that the hnRNP particles represent the native RNP complexes on which RNA processing occurs *in vivo* (37). In this paper we show that part of U2AF⁶⁵ and PTB [another polypyrimidine tract binding protein (38, 39)], and all of the nucleoplasmic phosphorylated forms of SR proteins detected by mAb 104 are integral components of the hnRNP particles. We further show that the structural integrity of hnRNP particles is correlated with variations in the phosphorylation levels of the SR proteins, indicating that the phosphorylation state of SR proteins may

Abbreviations: RNP, ribonucleoprotein; hnRNP, large nuclear ribonucleoprotein; U snRNA, uridine-rich small nuclear RNA; snRNP, small nuclear ribonucleoprotein; SR protein, Ser/Arg-rich protein; U2AF, U2 snRNP auxiliary factor; PTB, polypyrimidine tract binding protein; β -GP, β -glycero phosphate.

play a regulatory role in the assembly of the InRNP particles *in vivo*.

MATERIALS AND METHODS

Preparation of InRNP Particles. Nuclear supernatants enriched for InRNP particles were prepared from Syrian hamster or HeLa cells and fractionated in 15–45% sucrose gradients according to Sperling *et al.* (31) with modifications as described in refs. 33 and 34. For a second fractionation, gradient fractions corresponding to the 200S peak were combined, dialyzed, concentrated, and refractionated on a second sucrose gradient as described (32). The peak of 200S tobacco mosaic virus, sedimented in parallel gradients, was used as a reference.

Protein Analyses. For slot blot analysis, aliquots from sucrose gradient fractions were spotted directly onto a nitrocellulose membrane. For Western blot analysis, sucrose gradient fractions were concentrated, electrophoresed on an SDS/10% polyacrylamide gel and transferred to a nitrocellulose membrane. SR proteins were probed according to Zahler *et al.* (9) with mAb 104, which identifies phosphorylated epitopes of the SR proteins (11). Anti-SR protein mAb 104 was kindly provided by A. Zahler and M. Roth (Fred Hutchinson Cancer Research Center, Seattle). U2AF and PTB were probed using rabbit polyclonal antibodies (diluted 1:5000) and protein A peroxidase (diluted 1:3000). The antibodies, raised against the respective recombinant proteins, were kindly provided by J. Patton (Vanderbilt University, Nashville, TN). Stained proteins were visualized by chemiluminescence using the ECL commercial kit (Amersham).

RNA Analyses. For slot blot analysis of carbamoyl-P-synthetase, aspartate transcarbamylase, dihydro-orotase (CAD) RNA in fractions of sucrose gradients, aliquots were spotted directly onto nitrocellulose membranes and hybridized with a ^{32}P -labeled CAD subgenomic DNA probe according to Miriami *et al.* (33). For Northern blot analysis, RNA was prepared from immunoprecipitated material or from fractions of sucrose gradients by phenol extraction and ethanol precipitation. RNA was electrophoresed on a 7 M urea/10% polyacrylamide gel, transferred by electroblotting to a GeneScreen membrane and hybridized with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled RNA transcripts complementary to U1, U2, U4, U5, and U6 snRNAs according to Wu *et al.* (40). (The snDNA plasmids were

kindly provided by J. Gall, Carnegie Institution of Washington, Baltimore). The relative intensities of the U snRNAs do not reflect their stoichiometry in the 200S particles, since the probes used varied with respect to their hybridization efficiencies with the respective target RNAs.

Immunoprecipitations. The nuclear supernatant of Syrian hamster cells enriched in InRNP particles was treated with an anti-SR mAb, or with a nonrelevant control mAb, bound to protein G Sepharose beads according to Roth *et al.* (8), except that no linker antibody was used. RNA was released from the beads by phenol extraction and ethanol precipitation and analyzed by Northern blot as described above. Proteins were released by treatment with an SDS-containing gel sample buffer and analyzed by Western blot as described above. A similar procedure was used to analyze fractions from sucrose gradients. For these indirect immunoprecipitations we used mAb 1H4, an anti-SR mAb of the IgG type (K. M. Neugebauer and M. B. Roth, unpublished work), kindly provided by these authors. Similar qualitative results were obtained with mAb 104 (IgM); however, better yields were obtained with mAb 1H4.

RESULTS

The Nucleoplasmic Phosphorylated SR Proteins Sediment at the 200S Region in a Sucrose Gradient. Based on the presumed function of the SR proteins in pre-mRNA splicing (25) and on their tendency to interact with spliceosomal snRNPs through direct protein–protein interactions (17, 23), this family of proteins as a whole is expected to be associated with the RNA processing machinery *in vivo*. As a first step toward demonstrating this association, we show (Fig. 1) that SR proteins cosediment with U snRNPs at the 200S region in a sucrose gradient, where CAD RNA and several specific nuclear pre-mRNAs have been shown to sediment (31–34). For the experiment shown in Fig. 1, a nuclear supernatant enriched in RNP particles was prepared from Syrian hamster cells and fractionated on a sucrose gradient. Fractions corresponding to the 200S peak region of this gradient were combined, concentrated, and refractionated on a second sucrose gradient. The second fractionation was required, since after one fractionation U snRNPs are found associated with pre-mRNAs in 200S complexes, but also at the top of the

FIG. 1. SR proteins sediment at the 200S region in a sucrose gradient. Nuclear supernatant of Syrian hamster cells enriched in InRNP particles was fractionated in a 15–45% sucrose gradient. The 200S peak fractions were combined and refractionated on a second sucrose gradient. (Upper) The distribution of SR proteins across the second sucrose gradient as revealed by slot blot analysis with mAb 104 (11). (Lower) Northern blot analysis of the five spliceosomal U snRNAs present in fractions of the second sucrose gradient. The sedimentation of 200S tobacco mosaic virus, measured in a parallel gradient, was used as a reference.

sucrose gradients, where apparently free U snRNPs sediment (see ref. 35, and Fig. 4 in this paper). Northern blot analysis of the distribution of the spliceosomal U snRNAs across the second gradient shows now (Fig. 1 *Lower*) that all five spliceosomal U snRNAs (U1, U2, U4, U5, and U6) cosediment with CAD RNA (not shown) in a single peak that corresponds to purified 200S InRNP particles. Slot immunoblot analysis of the same fractions, using anti-SR mAb 104 (11), shows that the SR proteins sedimented in a peak that overlaps that of the U snRNAs (Fig. 1 *Upper*). Similar results were obtained when parallel analyses were performed on HeLa cells.

To analyze the distribution of individual SR proteins across the sucrose gradient, we performed a Western immunoblot analysis. For this purpose a nuclear supernatant of HeLa cells was fractionated on a sucrose gradient only once and the protein content of aliquots from each fraction were displayed on an SDS/polyacrylamide gel. Probing a blot of this gel with mAb 104 revealed a single peak for each SR protein at, or near, the 200S region of the sucrose gradient (Fig. 2). Similar results were obtained when analyses were performed on Syrian hamster cells. Interestingly, some of the bands corresponding to individual SR proteins are resolved into a few bands that differ slightly in their mobilities. These differences may reflect different phosphorylation states of individual SR proteins or the existence of additional members of the SR family. An additional group of high molecular weight proteins (>100 kDa), which are recognized by mAb 104, is also associated with the InRNP particles.

The sedimentation patterns of the SR proteins are strikingly different from those of the spliceosomal U snRNPs. While two populations of the U snRNPs can be distinguished: one associated with 200S InRNP particles, and another that sediment at the top of the gradient (see above), all of the mAb 104 detectable antigens are found only at the 200S region of the gradient. This feature is apparently unique to SR proteins, as the sedimentation patterns of another essential splicing factor, e.g., U2AF, and of PTB that competes with U2AF in specific cases (41), is similar to that of the snRNPs. Thus, a Western blot analysis of U2AF⁶⁵ (Fig. 3A) shows that this protein cosediments with 200S particles in a distinct peak, but also sediments with smaller complexes, or in a free form, at the top

FIG. 2. Individual SR proteins sediment at the 200S region in a sucrose gradient as revealed by Western blotting. Nuclear supernatant of the HeLa cells enriched in InRNP particles was fractionated in a 15–45% sucrose gradient only once. Aliquots from each fraction were electrophoresed on an SDS/10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with mAb 104. Lane SR, calf thymus SR proteins prepared as described by Zahler *et al.* (9).

FIG. 3. U2AF and PTB sediment at the 200S region in sucrose gradients. Nuclear supernatant of HeLa cells enriched in InRNP particles was fractionated in a 15–45% sucrose gradient. Aliquots from each fraction were electrophoresed on an SDS/10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-U2AF antibodies (A), and with anti-PTB antibodies (B *Upper*). Fractions corresponding to the 200S peak (fractions 8–12) were combined, refractionated on a second sucrose gradient, and probed with anti-PTB antibodies (B *Lower*).

of the gradient. Similar analysis of PTB (Fig. 3B, *Upper*) shows that most of this protein sediments in a peak corresponding to small complexes, which trails toward higher sedimentation values. To demonstrate the cosedimentation of PTB with 200S particles, fractions corresponding to the 200S region of the first gradient were combined and refractionated in a second gradient (Fig. 3B, *Lower*). A single peak of PTB can now be seen centered around fractions 10 and 11.

SR Proteins Are Associated with CAD RNA and U snRNPs in 200S InRNP Particles. The association of the SR proteins with the 200S InRNP particles was demonstrated by indirect immunoprecipitations of CAD RNA and of spliceosomal U snRNAs from the 200S peak region, by anti-SR mAbs. In a control experiment, we showed that a mAb directed against SR proteins, but not a nonrelevant mAb, specifically immunoprecipitated all spliceosomal U snRNAs and Sm proteins from a nuclear supernatant enriched in InRNP particles (data not shown). The distribution of immunoprecipitated CAD RNA (Fig. 4A) was monitored by RNA blot analysis using a CAD subgenomic probe (33). A single peak of immunoprecipitated CAD RNA, centered at 200S, can be seen. This pattern is similar to that of the SR proteins (see Fig. 1) and to that of CAD RNA, which had been present in the gradient fractions before precipitation (ref. 32 and data not shown). The U snRNAs that had been indirectly immunoprecipitated by the anti-SR mAbs were analyzed in a Northern blot using antisense RNA probes. Fig. 4B *Left* (Pellet) shows the snRNAs that had been immunoprecipitated from the bottom, 200S, and top fractions of the gradient (fractions 2, 10, and 20, respectively). For comparison, the U snRNAs that remained in solution are displayed in Fig. 4B *Right* (Supernatant). The usage of an RNP preparation that had been fractionated only once provided an internal control for this experiment, as apparently free U snRNPs are abundant at the top fractions of this gradient (see above). It can be seen that substantial amounts of all spliceosomal U snRNAs were found in the pellet of the 200S fraction, whereas only minute amounts of these RNAs were precipitated from the top fraction of the gradient—despite the high levels of U snRNAs present there. Noticeably, the relative amounts of the U snRNAs in the pellet of the top fraction differ from that in the corresponding supernatant. It is therefore possible that the U snRNA signals observed in the pellet of the top fraction reflect a specific interaction(s) of U snRNPs with individual SR proteins whose peaks extend to the top of the gradient. Implying the same

FIG. 4. SR proteins are associated with CAD RNA and U snRNPs in 200S InRNP particles. Nuclear supernatant of Syrian hamster cells enriched in InRNP particles was fractionated in a 15–45% sucrose gradient. Aliquots from each fraction were immunoprecipitated by anti-SR proteins mAb 1H4 bound to protein G Sepharose beads and RNA was prepared by phenol extraction and ethanol precipitation. (A) Distribution of indirectly immunoprecipitated CAD RNA as analyzed by slot blot hybridization with a CAD subgenomic DNA probe. (B) Northern blot analysis of indirectly immunoprecipitated U snRNAs in gradient fractions 2, 10, and 20 (Left lanes marked Bot, 200S and Top, respectively). The remaining fraction of U snRNAs in the respective supernatants is shown for comparison (Right).

possibility are differences in the relative amounts of U snRNAs that can be observed between the pellets of the 200S and top fractions. For example, the U5/U4 ratio in the pellet of the 200S fraction is much higher than that in the pellet of the top fraction, whereas in the corresponding unprecipitated fractions this ratio is similar. Specific reagents that recognize individual SR proteins should allow a more precise and unambiguous assignment of these specific interactions. These experiments, however, suggest the occurrence of specific interactions of the SR proteins with the pre-mRNA and the spliceosomal U snRNPs within the 200S InRNP particles.

The Structural Integrity of InRNP Particles Is Correlated with the Phosphorylation State of SR Proteins. Our observation that all of the mAb 104 detectable SR proteins in the nuclear supernatant of the cell are associated with InRNP particles, suggested that the structural integrity of the InRNP particles may depend on the phosphorylation state of SR proteins. Indeed, treatment of 200S InRNP particles with phosphatase PP1 γ , which has been shown to dephosphorylate most of the SR proteins (29), caused the dissociation of the particles and partial aggregation of SR proteins (data not shown). To test the effect of hyperphosphorylation of SR proteins on their association with InRNP particles, we took advantage of an SR protein kinase activity that is present in the nuclear supernatant. In Fig. 5 we show that this activity can be simply manifested when phosphatase activity is inhibited by the potent phosphatase inhibitor β -glycero-phosphate (β -GP). Partial phosphorylation of SR proteins can already be induced by incubation of a nuclear supernatant at 30°C (lane 2). However, incubation at 30°C in the presence of β -GP caused a substantial increase in the phosphorylation level of the SR proteins (compare lane 3 with lanes 1 or 2). This result indicates that the phosphorylation state of SR proteins is controlled by the balanced activities of phosphatase(s) and kinase(s). The inhibition of the phosphatase activity disrupts this balance and makes the kinase activity apparent. This balance of phosphatase(s) and kinase(s) is not affected by β -GP at 4°C, as no change in SR proteins phosphorylation was observed (data not shown).

FIG. 5. Nuclear supernatants enriched in 200S InRNP particles contain SR protein kinase activity. Aliquots from a nuclear supernatant (NS) prepared from HeLa cell nuclei were incubated for 1 hr as indicated, electrophoresed on an SDS/10% polyacrylamide gel, transferred to a nitrocellulose membrane and probed with mAb 104. Lane 1, untreated NS incubated at 4°C; lane 2, NS incubated at 30°C; lane 3, NS incubated at 30°C in the presence of 20 mM β -GP.

The effect of such kinase activity on the integrity of InRNP particles was demonstrated in the following experiment. Fractions corresponding to the 200S region of a sucrose gradient, in which a nuclear supernatant of HeLa cells had been fractionated, were combined and an aliquot was incubated at 30°C in the presence of ATP and β -GP. For controls, an identical aliquot was treated only with ATP; another one was incubated at 30°C without treatment; and a third aliquot was left at 4°C with no treatment. After incubation for 30 min, all samples were fractionated in sucrose gradients, and the distribution of SR proteins across each gradient was determined by immuno-slot blot analysis with mAb 104 (Fig. 6). Incubation without treatment and at low temperature caused no change in the sedimentation profile of the SR proteins, which peaks at

FIG. 6. Hyperphosphorylation of SR proteins is accompanied by dissociation of InRNP particles. Nuclear supernatant of HeLa cells was fractionated in a 15–45% sucrose gradient. Fractions corresponding to the 200S region (lanes 9–12) were combined, divided into four samples, and incubated for 30 min as indicated. All samples were refractionated in an identical sucrose gradient, and the distribution of SR proteins across the gradient was revealed by slot blot analysis with mAb 104. Incubations were as follows: 4°C, no additives (first row); 30°C, no additives (second row); 30°C, with 2 mM ATP (third row); and 30°C, with 2 mM ATP and 20 mM β -GP (fourth row).

200S (row marked 4°C). A similar sedimentation profile was obtained upon incubation at 4°C in the presence of ATP and β -GP (not shown). Raising the temperature to 30°C, in the absence or presence of ATP, resulted in a shift of the peak to a lower sedimentation value and in the appearance of SR proteins at the top of the gradient (rows marked 30°C and 30°C+ATP). Finally, after incubation at 30°C in the presence of both ATP and β -GP almost all of the SR proteins sedimented at the top of the gradient (row marked 30°C+ATP+ β -GP), presumably due to dissociation of the lnRNP particles. Sedimentation profiles similar to those shown in Fig. 6 were also obtained for the Sm antigens (not shown), thus substantiating this supposition. It can also be seen that, under these conditions, the integrated intensity of the SR protein signal increased substantially, as expected from the experiment shown in Fig. 5. We have thus shown that hyperphosphorylation of SR proteins was accompanied by disruption of the lnRNP particles. It is possible that this effect was caused by the hyperphosphorylation of the SR proteins, though the possibility that phosphorylation of other lnRNP proteins may have been responsible for this result, cannot be ruled out. Nevertheless, we argue that the former interpretation is highly probable, because SR proteins, which were shown to be involved in protein-protein and protein-RNA interactions within the spliceosome (reviewed in ref. 10), are likely to play a similar role in the lnRNP particles. Furthermore, changes in the phosphorylation state of the charged SR domain are expected to affect interactions involving SR proteins. The fact that the phosphorylated, mAb 104 detectable, SR proteins are associated exclusively with the lnRNP particles, imply that changes in their phosphorylation state will affect the structural integrity of the lnRNP particles.

DISCUSSION

We have demonstrated here that U2AF⁶⁵, PTB, and the SR protein family are integral components of 200S lnRNP particles, which are obtained by fractionation of nuclear supernatants, devoid of cytoplasmic contamination, in sucrose gradients. This study thus extends the list of essential splicing factors that, together with the five spliceosomal U snRNPs and undegraded endogenous pre-mRNAs, constitute the lnRNP particles. The composition of the lnRNP particles and the fact that they are isolated from mammalian nuclei in an apparently intact form, indicates that they faithfully represent the RNA processing machinery of the living cell. We have further shown that the structural integrity of lnRNP particles is sensitive to variations in the phosphorylation levels of the SR proteins, indicating that changes in the phosphorylation state of SR proteins may play a regulatory role in the assembly of lnRNP particles *in vivo*.

The sedimentation patterns of SR proteins across the sucrose gradients are strikingly different from those of the spliceosomal U snRNPs, including the Sm antigens, and even from SF53/4 (33, 36), U2AF⁶⁵, and PTB (Fig. 3). While the latter are found in two main populations: one in association with pre-mRNAs in 200S lnRNP particles and another in an apparently free form at the top of the gradient (33), the SR proteins recognized by mAb 104 are found only at the 200S region of the gradient (Fig. 2). Interestingly, the sedimentation profiles of individual SR proteins do not overlap. Thus, the peaks of the SRp70-75, SRp55, SRp40, and SRp20 overlap the U snRNPs peak at 200S. SRp40 exhibits a broader peak that extends to sedimentation values higher and lower than 200S. The peak of SRp30-35, which includes SRp30a (SF2/ASF, refs. 12 and 13) and SRp30b (PR264/SC35, refs. 14 and 15) is also broad, but it is shifted to a sedimentation value slightly lower than 200S. It is plausible that these minor variations in the sedimentation patterns of the SR proteins reflect dynamic structural changes in the lnRNP particles, which are manifested in the existence of subpopulations of lnRNP particles at

steady state. Such structural variations may be caused by differential associations of SR proteins with lnRNP particles and/or by variations in the phosphorylation levels of individual SR proteins while splicing of the pre-mRNA component of the lnRNP particles takes place.

Since mAb 104 recognizes only the phosphorylated form(s) of SR proteins (8, 11), it is evident that all of the nucleoplasmic amount of these antigens is associated with lnRNP particles. It seems therefore likely that the nuclear amount of these phosphorylated SR proteins is limiting for what is required for the assembly of the splicing machinery and for splicing efficiency. In contrast, U snRNPs and other splicing components, whose amount is in excess of what is required for splicing (42-44), sediment with 200S lnRNP particles but also at the top of the sucrose gradients in an apparently free form. The proposed limiting role of SR proteins in the assembly of the splicing machinery is consistent with the low levels of SR proteins found in nuclei (8). It is also in accordance with studies indicating the involvement of SR proteins in the early steps of spliceosome assembly (17, 19, 22, 26, 45); and with the role that single SR proteins play in committing specific pre-mRNAs to splicing (19).

Our finding that hyperphosphorylation of SR proteins within lnRNP particles can be induced by a phosphatase inhibitor indicates that phosphatase(s) and kinase(s) activities are also integral components of the particles. Further experiments, however, are required to determine whether these activities are specific to SR proteins. In any event, the structural integrity of lnRNP particles has been shown to be correlated with the phosphorylation state of the SR proteins, and thus appears to be maintained by balanced phosphorylation and dephosphorylation activities. Similar observations have been made *in vitro*, suggesting that both phosphorylation and dephosphorylation of SR proteins play important roles in splicing and splice site selection (46). The experiments reported here imply a possible role for the SR proteins, in regulating the structure and function of the lnRNP particles, through modulating their phosphorylation state by concerted phosphatase and kinase activities.

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