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A Discrete 3′ Region of U6 Small Nuclear RNA Modulates the Phosphorylation Cycle of the C1 Heterogeneous Nuclear Ribonucleoprotein Particle Protein†

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The C heterogeneous ribonucleoprotein particle (hnRNP) proteins bind to nascent pre-mRNA and may participate in assembly of the early prespliceosome. Ser/Thr phosphorylation of the C1 hnRNP protein in HeLa nuclear extracts regulates its binding to pre-mRNA (S. H. Mayrand, P. Dwen, and T. Pederson, Proc. Natl. Acad. Sci. USA 90:7764–7768, 1993). We have now further investigated the phosphorylation cycle of the C1 hnRNP protein, with emphasis on its regulation. Pretreatment of nuclear extracts with micrococcal nuclease eliminated the phosphorylation of C1 hnRNP protein, but pretreatment with DNase did not, suggesting a dependence on RNA. Oligodeoxynucleotide-targeted RNase H cleavage of U1, U2, and U4 small nuclear RNAs did not affect the phosphorylation of C1 hnRNP protein. However, cleavage of nucleotides 78 to 95, but not other regions, of U6 small nuclear RNA resulted in an inhibition of the dephosphorylation step of the C1 hnRNP protein phosphorylation cycle. This inhibition was as pronounced as that seen with the serine/threonine protein phosphatase inhibitor okadaic acid. C1 hnRNP protein dephosphorylation could be completely restored by the addition of intact U6 RNA. Add-back experiments with mutant RNAs further delineated the minimal region essential for C1 protein dephosphorylation as residing in nucleotides 85 to 92 of U6 RNA. These results illuminate a hitherto unanticipated function of U6 RNA: the modulation of a phosphorylation-dephosphorylation cycle of C1 hnRNP protein that influences the binding affinity of this protein for pre-mRNA. This newly revealed function of U6 RNA is likely to play a very early role in the prespliceosome assembly pathway, prior to U6 RNA's entry into the mature spliceosome's active center.

The C heterogeneous nuclear ribonucleoprotein particle (hnRNP) proteins are a highly conserved set of 42,000 to 44,000-molecular-weight proteins (3, 23, 37, 41) that associate with pre-mRNA at a very early posttranscriptional stage in vivo (2, 14, 15, 29, 55) and interact tightly and specifically with pre-mRNA in nuclear extract pre-mRNA splicing systems (35, 46). We have previously reported that the binding of the C hnRNP proteins to pre-mRNA is negatively regulated by hyperphosphorylation (34). A dynamic cycle of hnRNP C protein phosphorylation-dephosphorylation takes place in HeLa nuclear extracts, and inhibition of hnRNP C protein dephosphorylation by the serine/threonine protein phosphatase inhibitor okadaic acid leads to a markedly reduced affinity of C proteins for pre-mRNA (34).

In the present investigation, we have explored the possibility that the phosphorylation-dephosphorylation cycle of C hnRNP proteins is itself regulated and have focused on components of the splicing machinery as potential control elements. We identify a discrete 3′ region of U6 small nuclear RNA that modulates C hnRNP dephosphorylation.

MATERIALS AND METHODS

Nuclear extracts were prepared from HeLa cells as previously described (11). All extracts were prepared from cells harvested in logarithmic growth under the culture conditions detailed previously (40). Nuclear extracts (30%, vol/vol) were made 3.2 mM $MgCl_2$, 400 μM ATP, and 20 mM creatine phosphate prior to incubation with [γ-^{32}P]ATP and/or [γ-^{32}P]GTP at the concentrations specified in the figure legends. Where indicated, nuclear extracts were preincubated with the serine/threonine protein phosphatase inhibitor okadaic acid (1 μM; LC Services, Woburn, Mass.), with micrococcal nuclease (400 U/ml; Worthington Biochemical Corp., Freehold, N.J.) or with oligodeoxynucleotides (600 ng/μl unless otherwise specified; Integrated DNA Technologies, Coralville, Iowa) complementary to regions of U1, U2, U4, or U6 small nuclear RNA in the presence of 400 μM ATP, as detailed in the figure legends. Oligodeoxynucleotide-mediated cleavage of U1, U2, U4, and U6 RNAs by endogenous RNase H was monitored by electrophoresis of deproteinized nuclear extract RNA on 10% polyacrylamide–8.3 M urea gels followed by ethidium bromide staining (36, 49).

Following pretreatments as described above, the nuclear extracts were incubated with [γ-^{32}P]ATP and/or [γ-^{32}P]GTP as specified in the figure legends. C hnRNP proteins were isolated from the reactions by immunoaffinity selection on protein A-Sepharose-bound monoclonal antibody 4F4 (6) and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis as we have described in detail previously (34, 36) except that the acrylamide/*N*,*N*′-methylenebisacrylamide ratio in the gels was 74:1.

In some experiments, in vitro-transcribed human U6 small nuclear RNA or mutant U6 RNAs were added to nuclear extracts in which the endogenous U6 RNA had been destroyed by prior oligodeoxynucleotide-mediated RNase H cleavage. The wild-type U6 transcript was synthesized with T7 RNA polymerase from the human U6 plasmid, pHU6-1. pHU6-1 was constructed by PCR amplification of a human U6 RNA gene (pGEM/U6/dl-4) lacking nucleotides −4 to −1 5′ of the transcription start site (24), using two oligodeoxynucleotide primers, 5′-TGT AAT ACG ACT CAC TAT AGT GCT CGC TTC GGC AGC ACA-3′ and 5′-AAA AAT ATG GAA CGC TTC ACG AAT-3′. The PCR product was digested with *Hin*fI, blunt ended with Klenow fragment, and ligated into pGEM 3Zf(+). The resulting pGEM 3Zf(+)/T7 U6 DNA was uracil substituted, and oligodeoxynucleotide-mediated mutagenesis was performed with 5′ (5′-CGA CTC ACT ATA GGG GTG CTC GCT TCG-3′) and 3′ (5′-CGT TCC ATA TTT TTA AAT TCA GGT T-3′) U6 oligodeoxynucleotides. This produced pHU6-1, with a *Dra*I restriction site at the 3′ end of the U6 RNA coding sequence. Transcription of *Dra*I-cleaved pHU6-1 with T7 RNA polymerase produces a transcript corresponding to human U6 RNA with three additional G's at the 5′ end. A mutant U6 RNA containing nucleotides 1 to 92 was transcribed from a human plasmid, pHU6-2. pHU6-2 was constructed from pGEM 3Zf(+)/T7 U6 DNA (see above) and subjected to uracil substitution and oligodeoxynucleotide-mediated mutagenesis using two oligodeoxynucleotide primers, 5′ (5′-CGA CTC ACT ATA GGG GTG CTC GCT TCG-3′) and 3′ (5′-CAA ATT CGT GAA CGC GTT CCA TAT-3′). Transcription of *Mlu*I-cleaved pHU6-2 with T7 RNA polymerase produced a transcript corresponding to nucleotides 1 to 92 of human U6 RNA. The correct sequences of pHU6-1 and pHU6-2 were verified by dideoxy DNA sequencing. Where specific nucleotide

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† Dedicated to the memory of Kevin Van Doren.

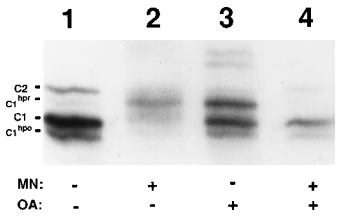

FIG. 1. Micrococcal nuclease eliminates basal phosphorylation of C1 hnRNP protein. HeLa nuclear extracts were incubated in the presence or absence of 400 U of micrococcal nuclease (MN) per ml in the presence of 1 mM $CaCl_2$ for 30 min at 30°C and then incubated with or without 1 μM okadaic acid (OA) for 20 min at 30°C. The samples were then made 3.2 mM $MgCl_2$, 400 μM ATP, 20 mM creatine phosphate, and 400 μCi of [γ-^{32}P]GTP per ml and incubated at 30°C for 30 min. C hnRNP proteins were immunoaffinity selected and analyzed by gel electrophoresis and autoradiography as detailed in Materials and Methods. The labeling of bands as C2, C1^{hpr}, C1, and C1^{hpo} is explained in the text.

positions in the transcripts of pHU6-1 and pHU6-2 are referred to in Results, the numbering begins with the fourth nucleotide from the 5′ end as nucleotide 1, so that the nucleotide positions stated correspond to those for native U6 RNA. A third mutant human U6 RNA, lacking nucleotides 75 to 84, was transcribed with SP6 RNA polymerase from plasmid SP6-U6ΔN7 (4). In experiments in which these U6 RNA transcripts were added back to extracts in which the endogenous U6 RNA had been cleaved by RNase H, the initial U6 oligodeoxynucleotide was destroyed by DNase I digestion (see the legend to Fig. 5). The completeness of oligodeoxynucleotide destruction by DNase was checked by assaying the integrity of the added-back U6 transcripts after subsequent incubation in the extracts.

RESULTS

Basal phosphorylation of C1 hnRNP protein is RNA-dependent. As a first step in exploring whether the phosphorylation cycle of C hnRNP proteins is in any way modulated by spliceosomal components, we examined the effects of prior treatment of nuclear extracts with micrococcal nuclease on subsequent C hnRNP protein phosphorylation. Figure 1, lane 1, shows the basal phosphorylation pattern of immunoaffinity-selected C hnRNP proteins after a 30-min incubation of nuclear extract under splicing-permissive conditions in the presence of [γ-^{32}P]GTP. Under these electrophoretic conditions, the ^{32}P-containing C1 and C2 hnRNP proteins were resolved into four distinct bands which, on the basis of previous work (34), we designated C1^{hpo} (for C1 hypophosphorylated), C1, C1^{hpr} (for C1 hyperphosphorylated), and C2. The relative autoradiographic intensities of the C1 and C1^{hpr} bands were significantly altered when the dephosphorylation step of the phosphorylation cycle was blocked by the serine/threonine protein phosphatase inhibitor okadaic acid, as shown in lane 3 of Fig. 1 (see also Fig. 2 in reference 34), with a much larger proportion of the ^{32}P now appearing in C1^{hpr} relative to C1.

As shown in lanes 2 and 4 of Fig. 1, when the nuclear extract was pretreated with micrococcal nuclease, the overall levels of C hnRNP protein phosphorylation observed after subsequent incubation with [γ-^{32}P]GTP were significantly reduced. This result indicates that nucleic acid is critically important for C hnRNP protein phosphorylation in the nuclear extracts. Since pretreatment of nuclear extracts with DNase I did not affect C hnRNP protein phosphorylation (data not shown), we conclude that the effect of micrococcal nuclease pretreatment (Fig. 1) reflects the importance of nuclear extract RNA, or RNAs, in C hnRNP phosphorylation.

Prompted by these results, we investigated the possibility that the integrity of spliceosomal small nuclear RNAs is critical for C hnRNP protein phosphorylation, mindful also of our previous finding that intact U1 and U2 snRNPs are required for the organization of C hnRNP proteins in a UV cross-linkable configuration on pre-mRNA (36).

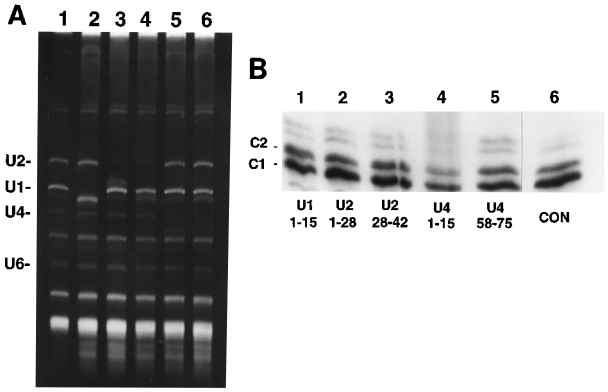

FIG. 2. Intact U1, U2, and U4 small nuclear RNAs are not required for phosphorylation of C hnRNP proteins. Nuclear extracts were incubated for 30 min at 30°C with oligodeoxynucleotides complementary to various sequences in U small nuclear RNAs in the presence of 400 μM ATP and then incubated for 10 min with DNase I at 100 μg/ml. At the end of the incubation, aliquots of each reaction were removed for extraction of RNA and subjected to electrophoresis in denaturing polyacrylamide gels and ethidium bromide staining to monitor the extent and selectivity of oligodeoxynucleotide-mediated cleavage. A parallel portion of each reaction was assayed for C hnRNP protein phosphorylation by subsequent incubation with [γ-^{32}P]ATP (150 μCi/ml) and [γ-^{32}P]GTP (150 μCi/ml). C hnRNP proteins were immunoaffinity selected and analyzed as for Fig. 1. (A) Analysis of oligodeoxynucleotide-mediated RNase H cleavages. Lane 1, no oligodeoxynucleotide; lane 2, oligodeoxynucleotide complementary to nucleotides 1 to 15 of U1 RNA; lane 3, oligodeoxynucleotide complementary to nucleotides 1 to 28 of U2 RNA; lane 4, oligodeoxynucleotide complementary to nucleotides 28 to 42 of U2 RNA; lane 5, oligodeoxynucleotide complementary to nucleotides 1 to 15 of U4 RNA; lane 6, oligodeoxynucleotide complementary to nucleotides 58 to 75 of U4 RNA. (B) Phosphorylated C hnRNP proteins from extracts preincubated with oligodeoxynucleotides as follows: lane 1, oligodeoxynucleotide complementary to nucleotides 1 to 15 of U1 RNA; lane 2, oligodeoxynucleotide complementary to nucleotides 1 to 28 of U2 RNA; lane 3, oligodeoxynucleotide complementary to nucleotides 28 to 42 of U2 RNA; lane 4, oligodeoxynucleotide complementary to nucleotides 1 to 15 of U4 RNA; lane 5, oligodeoxynucleotide complementary to nucleotides 58 to 75 of U4 RNA; lane 6, no-oligodeoxynucleotide control (CON). All oligodeoxynucleotides were used at a final concentration of 600 ng/μl except the U1 oligodeoxynucleotide which was used at 420 ng/μl.

U1, U2, U4, and U6 small nuclear RNAs are not required for C hnRNP protein phosphorylation, but U6 RNA is required for dephosphorylation. Reasonably likely candidates for the putative RNA or RNAs required for C hnRNP protein phosphorylation are the spliceosomal small nuclear RNAs U1, U2, U4, U5, and U6. Oligodeoxynucleotides complementary to regions of U1, U2, and U4 RNAs were incubated with nuclear extract under conditions in which endogenous RNase H cleaves the small nuclear RNA sites to which the oligodeoxynucleotide hybridizes. To verify that the oligodeoxynucleotides complementary to U1, U2, and U4 RNAs were indeed inducing cleavage, RNA from aliquots of oligodeoxynucleotide-treated extracts was analyzed by gel electrophoresis and ethidium bromide staining. Figure 2A shows that U1, U2, and U4 small nuclear RNAs were cleaved virtually 100% by their respective oligodeoxynucleotides. However, the cleavage of U1, U2, and U4 small nuclear RNAs had little or no effect on C1 hnRNP protein phosphorylation, as shown in Fig. 2B (C1 and C2 in lanes 6 versus lanes 1 to 5).

Figure 3A shows the extent of RNA cleavage induced by oligodeoxynucleotides complementary to three distinct regions of U6 RNA, nucleotides 19 to 29, 48 to 61, and 78 to 95. Densitometry of the negative of the ethidium bromide-stained gel revealed that the oligodeoxynucleotide complementary to nucleotides 78 to 95 induced cleavage of ~70% of the U6 RNA (Fig. 3A; compare lane 3 with the no-oligodeoxynucle-

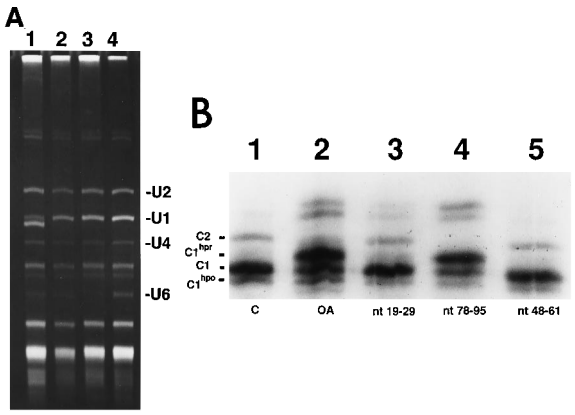

FIG. 3. A sequence near the 3′ end of U6 RNA is required for dephosphorylation of C1 hnRNP protein. Nuclear extracts were preincubated with an oligodeoxynucleotide (600 ng/μl) complementary to any one of three regions of U6 RNA and 400 μM ATP for 30 min at 30°C. Aliquots of each reaction were removed for analysis of U6 cleavage by electrophoresis and ethidium bromide staining as described in the legend to Fig. 2. C hnRNP protein phosphorylation, immunoselection, and electrophoresis were carried out as described for Fig. 1. (A) Analysis of oligodeoxynucleotide-mediated RNase H cleavages. Lane 1, oligodeoxynucleotide complementary to nucleotides 19 to 29 of U6 RNA; lane 2, oligodeoxynucleotide complementary to nucleotides 48 to 61 of U6 RNA; lane 3, oligodeoxynucleotide complementary to nucleotides 78 to 95 of U6 RNA; lane 4, no oligodeoxynucleotide. (B) C hnRNP protein phosphorylation reactions. Lane 1, phosphorylated C hnRNP proteins from a control (C) extract; lane 2, phosphorylated C hnRNP proteins from an extract incubated with okadaic acid (OA; 1 μM) but no oligodeoxynucleotide; lane 3, phosphorylated C hnRNP proteins from a nuclear extract incubated with an oligodeoxynucleotide complementary to nucleotides (nt) 19 to 29 of U6 RNA; lane 4, phosphorylated C hnRNP proteins from a nuclear extract incubated with an oligodeoxynucleotide complementary to nucleotides 78 to 95 of U6 RNA; lane 5, phosphorylated C hnRNP proteins from a nuclear extract incubated with an oligodeoxynucleotide complementary to nucleotides 48 to 61 of U6 RNA.

otide control in lane 4). As shown in Fig. 3B, lane 4, cleavage of U6 by this oligodeoxynucleotide resulted in a marked change in the pattern of C1 hnRNP protein phosphorylation, namely, the pronounced appearance of the $C1^{hpr}$ band. In fact, the ratio of ^{32}P in $C1^{hpr}$ relative to C1 seen after cleavage of U6 nucleotides 78 to 95 was very similar to that observed after okadaic acid treatment (Fig. 3B, lane 2). This effect of U6 cleavage at nucleotides 78 to 95 on C1 protein hyperphosphorylation was seen consistently in numerous experiments. In contrast, cleavage of other regions of U6 RNA did not produce this effect. An oligodeoxynucleotide complementary to nucleotides 19 to 29 of U6 RNA induced extensive cleavage (estimated by densitometry to be 75%; Fig. 3A, lane 1), yet there was no effect on C1 hnRNP protein phosphorylation (Fig. 3B, lane 3). The third U6 oligodeoxynucleotide, complementary to nucleotides 48 to 61, induced approximately 45% cleavage of U6 RNA (Fig. 3A, lane 2) but did not affect C1 protein phosphorylation Fig. 3B, lane 5).

Over the course of several experiments, the extent of U6 RNA cleavage induced by the oligodeoxynucleotide complementary to nucleotides 78 to 95 ranged from 50 to 100%. Interestingly, there was a general correlation between the extent of U6 cleavage by this oligodeoxynucleotide and the degree of the C1 hnRNP protein phosphorylation effect: the more extensive the U6 cleavage, the greater the extent of C1 protein hyperphosphorylation. In contrast, the oligodeoxynucleotide complementary to nucleotides 19 to 29 induced U6 cleavages ranging from 70 to 100%, but these extensive cleavages never influenced C1 protein phosphorylation.

As shown in Fig. 4, the region of U6 RNA at which oligodeoxynucleotide-targeted RNase H cleavage leads to an inhibition of C1 hnRNP protein dephosphorylation (nucleotides 78 to 95) includes a sequence that base pairs with the 5′ end of U2 RNA (9, 54). However, this interaction with U2 RNA is not required for U6's role in C1 protein dephosphorylation, since oligodeoxynucleotide-mediated cleavage of nucleotides 1 to 28 of U2 RNA did not influence the results (Fig. 2B, lane 2). In addition, when cleavage of nucleotides 1 to 28 of U2 RNA was followed by cleavage of U6 nucleotides 78 to 95, the typical inhibition of C1 protein dephosphorylation was observed (data not shown). These results indicate that the effect of U6 RNA on C1 protein dephosphorylation does not accompany or require pre-mRNA splicing. This point is reinforced by the observation that oligodeoxynucleotide-mediated cleavage of a region of U4 RNA (nucleotides 58 to 75) that base pairs with U6 RNA in the active spliceosome had no effect on C1 protein dephosphorylation (Fig. 2B, lane 5).

To investigate whether the 3′ end of U6 RNA might also modulate the phosphorylation of other splicing-related proteins, experiments similar to those in Fig. 3 were carried out with a monoclonal antibody to a family of the SR proteins (57). Oligodeoxynucleotide-mediated cleavage of nucleotides 78 to 95 of U6 RNA did not generate hyperphosphorylated forms of any of the several proteins selected by this antibody (data not shown).

To determine if the inhibition of C1 hnRNP protein dephosphorylation brought about by oligodeoxynucleotide-mediated RNase H cleavage of nucleotides 78 to 95 of U6 RNA was reversible, T7-transcribed U6 RNA was added back to the

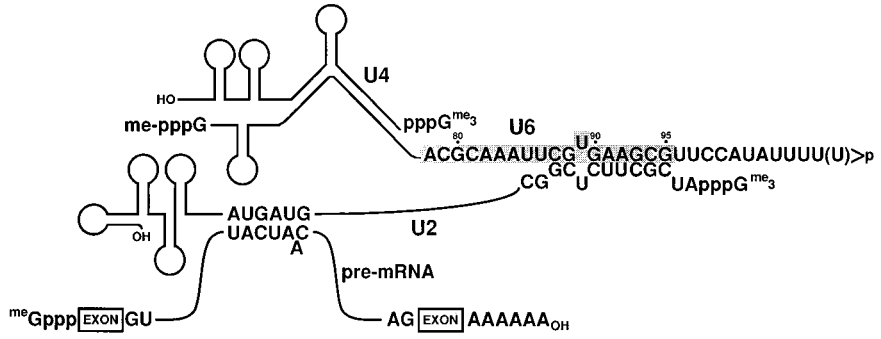

FIG. 4. Pre-mRNA–U2–U4–U6 RNA interactions. (modified from reference 9 in the context of the present results.) The region of U6 RNA at which oligonucleotide-mediated RNase H cleavage inhibits C1 protein dephosphorylation (nucleotides 78 to 95) is shaded. ^{me}Gppp, 7-methylguanosine-5′-triphosphate; $pppG^{me}{}_3$, 2,2,7-trimethylguanosine-5′-triphosphate; me-pppG, γ-methyl group esterified to the pppG 5′ terminus of U6 RNA (45); >p, cyclic-2′,3′ phosphate (25).

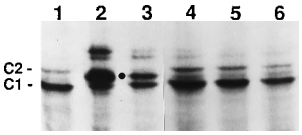

FIG. 5. Restoration of C1 hnRNP protein dephosphorylation by wild-type and mutant U6 RNAs. Nuclear extracts containing 400 μM ATP as usual were incubated with or without okadaic acid (1 μM) and with or without an oligodeoxynucleotide complementary to nucleotides 78 to 95 of U6 RNA (600 ng/μl) for 30 min at 30°C. After incubation with the U6 oligodeoxynucleotide $MgCl_2$ was added to 2 mM and DNase I was added to 3.7 U/μl, and the incubation continued for another 60 min at 37°C. This extract was made 3.2 mM $MgCl_2$, 20 mM creatine phosphate, and 40 μg of tRNA per ml and was then divided into four equal portions. One was incubated without further additions for 15 min at 30°C. To another, T7-transcribed human U6 RNA was added to a concentration of 20 ng/μl, and the incubation continued for 15 min at 30°C. To another, human U6 RNA lacking nucleotides 75 to 84 was added to 20 ng/μl, and the incubation was continued for another 15 min at 30°C. To another, human U6 RNA lacking nucleotides 93 to 106 was added to 20 ng/μl, and the incubation was continued for 15 min at 30°C. All of the reaction mixtures were then incubated for an additional 30 min at 30°C in the presence of [γ-^{32}P]ATP (150 μCi/ml) and [γ-^{32}P]GTP (150 μCi/ml). C hnRNP proteins were immunoselected and displayed by gel electrophoresis as in Fig. 1. Lane 1, untreated extract (no oligodeoxynucleotide or added-back U6 RNA); lane 2, extract incubated with 1 μM okadaic acid (but no oligodeoxynucleotide or added-back U6 RNA); lane 3, extract incubated with an oligodeoxynucleotide complementary to nucleotides 78 to 95 of U6 RNA (U6 oligodeoxynucleotide) but no added-back RNA; lane 4, extract incubated with U6 oligodeoxynucleotide followed by DNase treatment and incubation with wild-type U6 RNA; lane 5, extract incubated with U6 oligodeoxynucleotide followed by DNase treatment and incubation with U6 RNA lacking nucleotides 75 to 84; lane 6, extract incubated with U6 oligodeoxynucleotide followed by DNase treatment and incubation with U6 RNA lacking nucleotides 93 to 106. The dot between lanes 2 and 3 denotes the hyperphosphorylated C1 hnRNP protein.

oligodeoxynucleotide-treated extract after DNase destruction of the oligodeoxynucleotide. As shown in Fig. 5, lane 4, the addition of T7-transcribed U6 RNA restored the dephosphorylation of C1 hnRNP protein (compare with control in lane 1 and the usual effect of cleavage of U6 nucleotides 78 to 95 in lane 3). Addition of mutant U6 RNAs lacking nucleotides 75 to 84 (Fig. 5, lane 5) or nucleotides 93 to 106 (Fig. 5, lane 6) also restored dephosphorylation of C1 protein to an extent comparable to that seen in control extracts or ones to which wild-type U6 was added back. These results illuminate nucleotides 85 to 92 (or nucleotides therein) as the U6 RNA domain that modulates the dephosphorylation of the C1 hnRNP protein.

DISCUSSION

We have discovered a hitherto unexpected aspect of C1 hnRNP protein–pre-mRNA interaction dynamics, namely, an involvement of U6 RNA. This newly recognized function of U6 RNA is likely to temporally precede the established role of U6 in pre-mRNA splicing. In fact, our results demonstrate that U6 RNA's role in C1 hnRNP protein dephosphorylation does not require concurrent splicing, since the same role of U6 RNA in C1 protein dephosphorylation is observed in nuclear extracts in which splicing has been inactivated by oligodeoxynucleotide-mediated RNase H cleavage of U2 RNA. These observations suggest that before its entry into the active spliceosome (as the U4-U6 intermolecular complex), U6 RNA interacts with the dynamic phosphorylation machinery of the C1 hnRNP protein. The observed effect of U6 RNA on C1 protein dephosphorylation would be expected, on the basis of our previous results, to in turn facilitate the interaction of C1 protein with pre-mRNA (34).

It is possible that this envisioned early role of U6 RNA in suppressing C1 hnRNP protein hyperphosphorylation (and thus facilitating C1 protein binding to pre-mRNA) is reversed once U6 becomes integrated into the spliceosome's active center. Indeed, the splicing-requisite association of the 5′ end of U2 RNA with the 3′ end of U6 RNA (9, 54) might inactivate this U6 domain as regards C1 protein dephosphorylation. The consequence would be an accumulation of hyperphosphorylated C1 protein (just as when the U6 3′ domain is eliminated via oligodeoxynucleotide-mediated RNase H cleavage in our experiments) and this, in turn, would reduce the affinity of C1 protein for the pre-mRNA as we have shown (34). This postulated cycle of C1 hnRNP protein behavior is entirely compatible with the facts that this protein associates with pre-mRNA at a very early posttranscriptional stage (2, 14, 15, 29, 55), is clustered at the 3′ end of introns in splicing permissive nuclear extracts (46), and may be required for splicing (7, 13, 44). Indeed, there can be little doubt that at some point along the dynamic prespliceosome assembly pathway (27, 38, 51), the C hnRNP proteins must be actively dissociated from pre-mRNA in the vicinity of the spliceosome's active center, presumably having contributed up to then in shaping the pre-mRNA into a structural configuration conducive to splicing (see Discussion in reference 36). If allowed to remain bound to pre-mRNA beyond this stage in the spliceosome, the continual presence of C hnRNP proteins might compete with site-specific pre-mRNA binding of other proteins critical for splicing. Indeed, as we have previously emphasized (36, 50), the intron polypyrimidine tract region at which the C (and A1 and D) hnRNP proteins appear to be concentrated (46) is also the binding site for other proteins important for splicing such as U2AF (58), p62 (17, 50), and other proteins (18, 26, 48), suggesting a highly dynamic and complex sequential replacement of C hnRNP proteins at this site.

The 3′ domain of U6 RNA that we have implicated as a positive effector in the dephosphorylation of hyperphosphorylated C1 hnRNP protein is nucleotides 85 to 92 (or an even shorter portion thereof). This rules out as a factor in C1 hnRNP protein dephosphorylation the terminal cyclic-2′,3′ phosphate which is present on a major fraction of U6 RNA (25). Although our results establish that nucleotides 85 to 92 of U6 are clearly necessary for the dephosphorylation of hyperphosphorylated C1 hnRNP protein, we do not yet know if these nucleotides are sufficient. Indeed, we found that an oligoribonucleotide corresponding to nucleotides 78 to 95 of U6 RNA did not restore dephosphorylation of hyperphosphorylated C1 hnRNP protein after oligodeoxynucleotide-mediated RNase H cleavage of endogenous U6 RNA at nucleotides 78 to 95, in contrast to full-length U6 RNA, which fully restored dephosphorylation (Fig. 5). This finding again raises the possibility that nucleotides 85 to 92 of U6 RNA are necessary but not sufficient for promoting dephosphorylation of hyperphosphorylated C1 hnRNP protein. It is possible that the region of U6 RNA that we have identified in this investigation modulates C hnRNP protein phosphorylation-dephosphorylation as a switch, in turn depending on the spatial availability of this U6 region to C hnRNP protein in the spliceosome, since the intra- and intermolecular conformation of U6 RNA itself is highly dynamic during the spliceosome assembly-catalysis cycle (27, 38, 51, 52). Finally, it is to be emphasized that our results do not necessarily demonstrate that the 3′ end of U6 is itself the direct agent of the C1 phosphorylation reaction's modulation. It is entirely possible that U6 participates via the involvement of an intermediate factor or factors yet to be defined.

A stem-bulge structure in U6 RNA, involving nucleotides 27 to 46 and 83 to 101, has been identified as the binding site for the influenza virus NS1 protein (43). Since this protein inhibits splicing and nuclear export of host mRNAs, it is intriguing to

consider the possibility that this effect is related to an inhibitory action of NS1 protein on C1 hnRNP dephosphorylation, via its binding to the domain we have identified here (i.e., nucleotides 85 to 92). In this respect, the influenza virus NS1 protein could be envisioned as acting like our oligodeoxynucleotide-mediated RNase H cleavage of U6 nucleotides 78 to 95.

The regulation of hnRNP protein activity in the spliceosome by serine/threonine phosphorylation-dephosphorylation may be a general phenomenon. In addition to our findings with respect to dephosphorylation-mediated C hnRNP binding to pre-mRNA (34) and the role of U6 RNA in the dephosphorylation of hyperphosphorylated C1 hnRNP protein (this investigation), the serine/threonine phosphorylation level of another hnRNP protein, A1, has been correlated with its in vitro nucleic acid strand-annealing activity (8, 22). Moreover, the SR family of nuclear proteins, essential for initial recognition of the 5′ splice site by U1 snRNP during the commitment step of spliceosome assembly (21, 32, 33, 57), are known to be phosphorylated (19, 20). Since the A1 hnRNP protein and SR proteins operate as antagonists in 5′ splice site selection (31, 32, 56), it is conceivable that the balance is modulated by their respective phosphorylation states.

hnRNP protein phosphorylation may have even broader implications. Although presently based solely on in vitro phosphorylation studies, the following investigations point to an emerging connection between hnRNP protein phosphorylation and cellular functions. First, the phosphorylation of the A2 and C hnRNP proteins has been shown to be negatively regulated by nuclear calmodulin (5), suggesting a linkage with Ca^{2+}-mediated nuclear signalling events (1, 12, 28). In addition, the tyrosine phosphoproteins p62 and p68, which are associated with the $p21^{ras}$ GTPase-activating protein, display extensive sequence similarity to known hnRNP proteins (16, 47, 53), and tyrosine phosphorylation of an A/B-type hnRNP protein modulates its RNA binding activity (42), suggesting that certain hnRNP proteins may be targets for nuclear tyrosine phosphorylation-mediated signaling events (10). Finally, serine/threonine phosphorylation of the K hnRNP protein (30) is stimulated by the κB enhancer motif (39), raising the additional, novel possibility of a transcriptional role of hnRNP proteins. Clearly, the phosphorylation of hnRNP proteins is emerging as a most exciting aspect of their functional roles in gene expression.

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