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Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism

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Splicing of mRNA precursors (pre-mRNA) is preceded by assembly of the pre-mRNA with small nuclear ribonucleoprotein particles (snRNPs) and protein factors to form a spliceosome. Here we show that stimulating Ser/Thr-specific protein dephosphorylation selectively inhibits an early step during mammalian spliceosome assembly. Treatment of HeLa nuclear splicing extracts with human protein phosphatase 1 (PP1) expressed in *Escherichia coli*, or PP1 purified from rabbit skeletal muscle, prevents pre-spliceosome E complex (early complex) formation and stable binding of U2 and U4/U6.U5 snRNPs to the pre-mRNA. PP1 does not inhibit splicing catalysis if added after spliceosome assembly has taken place. Addition of purified SR protein splicing factors restores spliceosome formation and splicing to PP1-inhibited extracts, consistent with SR proteins being targets regulated by phosphorylation. These data extend earlier observations showing that splicing catalysis, but not spliceosome assembly, is blocked by inhibiting protein phosphatases. It therefore appears that pre-mRNA splicing, in common with other biological processes, can be regulated both positively and negatively by reversible protein phosphorylation.

Key words: protein phosphatase/protein phosphorylation/RNA splicing/spliceosome assembly/SR proteins

Introduction

Splicing of nuclear mRNA precursor transcripts (pre-mRNA) involves a two-step reaction mechanism and takes place in a dedicated complex termed a spliceosome (for reviews, see Green, 1991; Guthrie, 1991; Ruby and Abelson, 1991; Moore *et al.*, 1993; Rymond and Rosbash, 1993). The function and composition of spliceosomes have been studied in both mammalian and yeast *in vitro* splicing systems. In both systems they comprise three major RNA–protein subunits, the U1, U2 and U4/U6.U5 small nuclear ribonucleoprotein particles (snRNPs; reviewed by Guthrie and Patterson, 1988; Lührmann *et al.*, 1990), and additional non-snRNP protein splicing factors (reviewed by Lamm and Lamond, 1993). Spliceosomes assemble on pre-mRNA substrates in a stepwise manner, involving the formation of distinct pre-splicing complexes that represent functional intermediates in the pathway. The order of assembly appears to be obligatory, with U1 snRNP binding to the 5′ splice site, U2 snRNP interacting with the 3′ end of the intron prior to U4/U6.U5 assembly (reviewed by Lamond, 1993; Moore *et al.*, 1993). In yeast and mammals, the 5′ and 3′ splice sites are recognized and functionally associated with one another in one of the earliest characterized pre-splicing complexes containing U1 snRNP, but not U2 or U4/U6.U5 snRNPs (Reed, 1990; Michaud and Reed, 1991, 1993; Rosbash and Seraphin, 1991; Jamison *et al.*, 1992).

As well as the snRNP subunits, a number of non-snRNP protein splicing factors play important roles during mammalian spliceosome assembly. These include a group of proteins that share a common motif consisting of repeating serine–arginine dipeptides (Birney *et al.*, 1993). There is evidence that most, if not all, proteins with the SR motif are phosphorylated *in vivo* (Wooley *et al.*, 1988; Roth *et al.*, 1990; Woppmann *et al.*, 1993; Gui *et al.*, 1994). A subset of SR-containing splicing factors, including SRp20, ASF/SF2, SC-35, SRp40, SRp55 or SRp75, are recognized through a shared phosphoepitope by the same monoclonal antibody, called mAb104 (Roth *et al.*, 1990, 1991; Zahler *et al.*, 1992). This group of proteins can be co-purified using a two-step salt precipitation method (Zahler *et al.*, 1992). The characteristic serine–arginine repeat motif is also found in several other splicing factors that are not detected by mAb104 and do not co-purify with the other SR proteins. These include both subunits of the non-snRNP splicing factor U2AF (i.e. $U2AF^{65}$ and $U2AF^{35}$) and the U1 snRNP-specific 70 kDa protein (Theissen *et al.*, 1986; Query and Keene, 1987; Spritz *et al.*, 1987; Zamore *et al.*, 1992; Zhang *et al.*, 1992). To distinguish between these SR-containing splicing factors, we will refer to the proteins recognized by mAb104 as SR104 proteins.

Although its detailed function in the splicing mechanism is not yet known, mutational studies have shown that the SR domain in $U2AF^{65}$ and in ASF/SF2 is required for their activity as essential splicing factors (Zamore *et al.*, 1992; Caceres and Krainer, 1993; Zuo and Manley, 1993). U2AF, SR104 proteins and U1 snRNP are all required for spliceosome assembly or the stabilization of early pre-splicing complexes (Chabot and Steitz, 1987; Frendewey *et al.*, 1987; Zillmann *et al.*, 1987; Barabino *et al.*, 1990; Krainer *et al.*, 1990; Fu *et al.*, 1992; Mayeda *et al.*, 1992; Zahler *et al.*, 1992, 1993; Zamore *et al.*, 1992). For example, SR104 proteins can commit pre-mRNAs to the splicing pathway (Fu, 1993). ASF/SF2 has also been shown to cooperate with U1 snRNP in binding pre-mRNA (Khotz *et al.*, 1994), and protein–protein interaction between SC35 (ASF/SF2) and other SR proteins has been demonstrated (Wu and Maniatis, 1993).

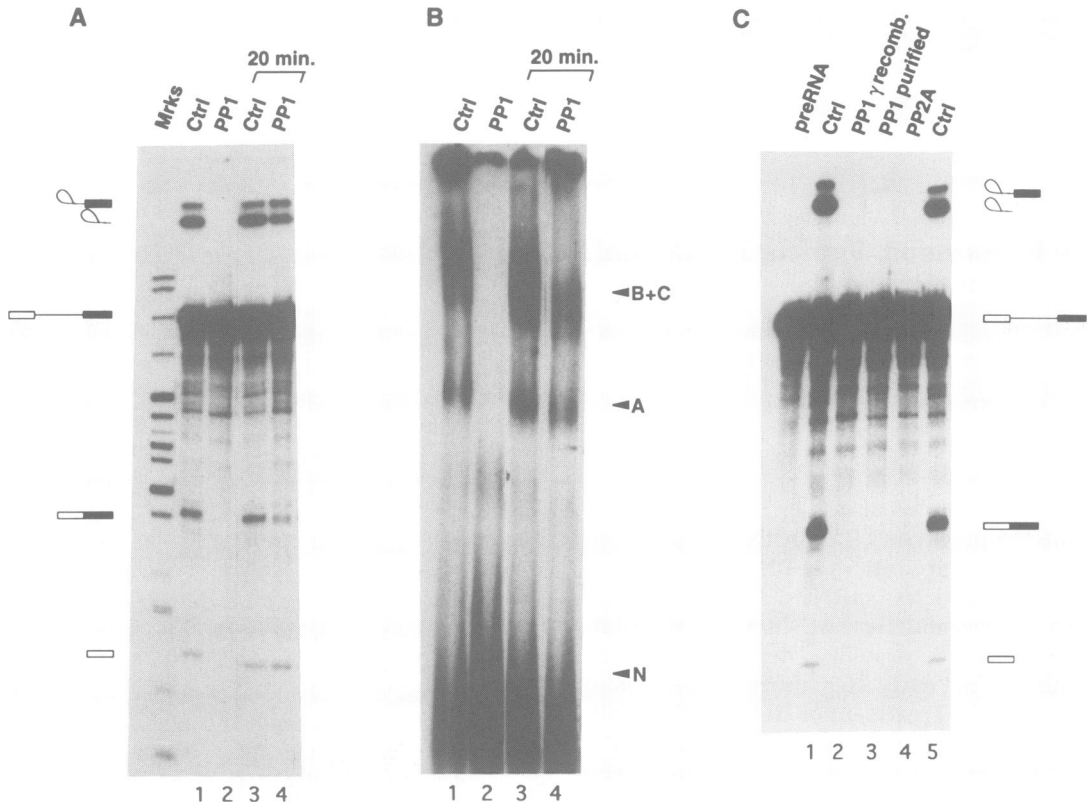

Fig. 1. Protein phosphatases inhibit pre-mRNA splicing and splicing complex formation. A ^{32}P-labelled Ad1 pre-mRNA substrate was incubated for 120 min at 30°C under standard splicing conditions in the absence or presence of recombinant or purified catalytic subunits of Ser/Thr specific protein phosphatases. (**A**) and (**B**) The lanes marked 'Ctrl' correspond to splicing reactions carried out in the absence of phosphatase (lanes 1 and 3). In lane 2, human PP1γ expressed in *E.coli* (0.1 U/μl) was added to the assay from the beginning of the reaction. In lanes 4, PP1γ (0.1 U/μl) was added 20 min after the start of the reaction. (**C**) Human PP1γ expressed in *E.coli* (0.15 U/μl; lane 2), purified PP1 catalytic subunits from rabbit skeletal muscle (0.5 U/μl; lane 3) and PP2A (0.7 U/μl; lane 4) were added at the beginning of the reaction. 'Ctrl' (lanes 1 and 4), no phosphatase was added. (A) and (C) The splicing products of Ad1 pre-mRNA were resolved on a 10% polyacrylamide/8 M urea gel. The structures of the pre-mRNA, splicing intermediates and products are indicated by cartoons with exons indicated as solid boxes and introns as lines. Markers are end-labelled *Msp*I-digested pBR322 fragments ('Mrks'). (B) Spliceosome complexes were separated on an agarose–polyacrylamide composite gel. Pre-spliceosome (A complex) and spliceosome complexes (B and C complexes) are indicated with arrows. 'N' represents non-specific complexes.

Recent data have indicated that inhibiting protein dephosphorylation on serine or threonine residues blocks the catalytic steps of splicing, but not spliceosome assembly (Mermoud *et al.*, 1992; Tazi *et al.*, 1992, 1993). HeLa nuclear splicing extracts contain both protein phosphatase 1 (PP1) and PP2A activities (Mermoud *et al.*, 1992). When these activities are inhibited by treatment with the specific phosphatase inhibitors okadaic acid, tautomycin or microcystin-LR, inactive spliceosomes are formed containing U1, U2 and U4/U6.U5 snRNPs (Mermoud *et al.*, 1992; Tazi *et al.*, 1992). Thiophosphorylation of the U1 snRNP-specific 70 kDa protein causes a similar effect (Tazi *et al.*, 1993). Splicing can be reactivated in extracts treated with phosphatase inhibitors by adding exogenous catalytic subunits of PP1 or PP2A (Mermoud *et al.*, 1992). There is also evidence that forms of PP1 and PP2A may differentially affect the first and second catalytic steps of splicing, indicating that at least two, and possibly more, separate dephosphorylation events are required for catalysis.

Here we show that stimulating specific Ser/Thr protein dephosphorylation events inhibits spliceosome formation at an early step of the assembly pathway in HeLa splicing extracts. We also present evidence that the phosphorylation state of the SR104 family of protein splicing factors may be crucial for their function in spliceosome assembly.

Results

Protein phosphatases inhibit spliceosome assembly

The effects of Ser/Thr-specific protein phosphatases on pre-mRNA splicing were investigated in HeLa nuclear extracts (Figure 1). Treatment of the extract with human PP1, isoform γ, expressed in *Escherichia coli* and added from the beginning of the assay, potently inhibits splicing (Figure 1A, lane 2). A parallel analysis revealed that PP1γ inhibited early steps in spliceosome assembly (Figure 1B). Lane 1 (panel B) shows the typical pre-splicing complex (A complex) and spliceosomes (B + C complexes) formed in a control splicing assay which are abolished in extracts treated with PP1γ (Figure 1B, lane 2). All pre-mRNAs tested were similarly inhibited by PP1γ in a concentration-dependent manner (data not shown). No spliced products were observed even after long incubation times, indicating that the inhibitory effect does not reflect a reduction in the kinetics of spliceosome assembly (data not shown). Inhibition of splicing was also observed using PP1 catalytic

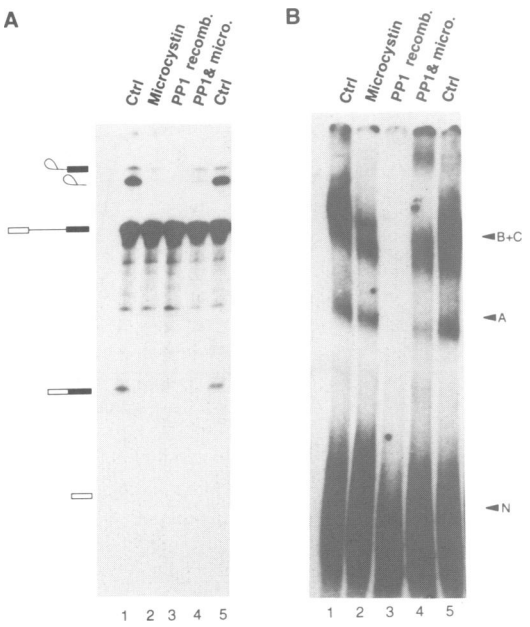

Fig. 2. The phosphatase-mediated block of spliceosome formation is sensitive to phosphatase inhibitors. The inhibition of splicing complex assembly caused by phosphatase treatment was prevented when PP1γ was pre-treated with microcystin-LR. (**A**) and (**B**) Lanes 1 and 5 show control splicing reactions with Ad1 pre-mRNA carried out in the absence of inhibitor. The splicing extract was treated with either microcystin-LR (0.6 μM; lane 2) or with with PP1γ (0.1 U/μl; lane 3). Lane 4 corresponds to the addition of pre-mixed PP1γ/microcystin-LR to the splicing reaction. (A) shows the separation of RNA intermediates and products on a denaturing gel, while (B) shows a parallel analysis of splicing complex assembly on a native agarose–polyacrylamide composite gel.

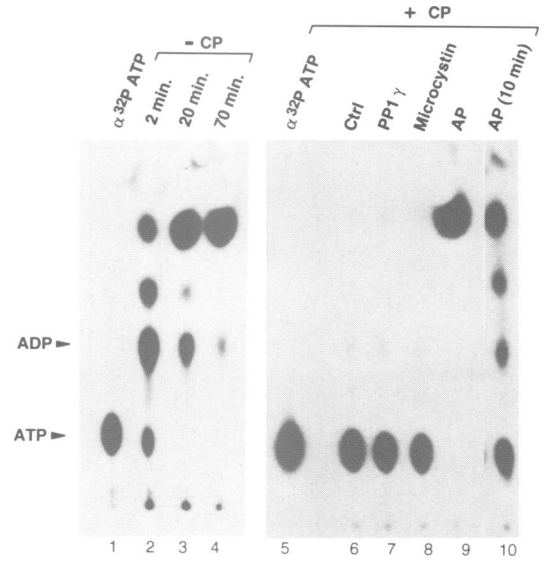

Fig. 3. ATP levels are stable in extracts treated with PP1γ or microcystin-LR. TLC analysis of α-^{32}P-labelled ATP incubated in HeLa nuclear extract under splicing conditions either in the absence or presence of splicing inhibitors. Lanes 1 and 5, [α-^{32}P]ATP; lanes 2–4 correspond to different time points of a splicing reaction incubated without exogenous cold ATP or creatine phosphate (CP). ATP levels are stable in a standard control splicing reaction (lane 6) and in extracts where splicing was blocked by treatment with either human PP1γ expressed in *E.coli* (0.3 U/μl; lane 7) or the phosphatase inhibitor microcystin-LR (1 μM; lane 8), but not in the presence of alkaline phosphatase (0.05 U/μl; lanes 9 and 10). The samples of the standard splicing reactions including CP were analysed after 1 h of incubation, the sample treated with alkaline phosphatase was also analysed at 10 min (lane 10). Parallel analysis confirmed that splicing was blocked in the extracts treated with PP1γ (lane 7), microcystin-LR (lane 8) and alkaline phosphatase (lanes 9 and 10), but not in the untreated control sample (lane 6; data not shown). The position of ATP and ADP is indicated with arrows; the identities of the other hydrolysis products were not investigated further.

subunits purified from rabbit skeletal muscle (Figure 1C, lane 3). Similarly, the catalytic subunit of PP2A, which has overlapping substrate specificity with PP1, also blocked splicing (Figure 1C, lane 4) and spliceosome assembly (data not shown).

When PP1γ was added to the splicing assay after 20 min, catalysis (Figure 1A, lane 4) and complex assembly (Figure 1B, lane 4) were still observed. Parallel analysis confirmed that at this time spliceosome assembly, but not the first catalytic step of splicing, has occurred (data not shown). The simplest interpretation of this result is that dephosphorylation events inhibit spliceosome assembly, but do not block splicing if complex formation has already occurred.

If inhibition of spliceosome assembly is caused by protein dephosphorylation, it should be alleviated by pre-treatment of the phosphatase with a specific phosphatase inhibitor. To test this, control reactions (Figure 2A and B, lanes 1 and 5) were compared with splicing reactions in the presence of the phosphatase inhibitor microcystin-LR (Figure 2A and B, lanes 2) or PP1γ (Figure 2A and B, lanes 3) and with reactions containing pre-mixed PP1γ/microcystin-LR (Figure 2A and B, lanes 4). Microcystin-LR is a specific phosphatase inhibitor that binds irreversibly to the catalytic subunits of PP1 and PP2A (MacKintosh *et al.*, 1990). It inhibits splicing, but allows assembly of splicing complexes (Mermoud *et al.*, 1992; Figure 2A and B, lanes 2). In contrast, addition of PP1γ abolishes splicing (Figure 2A, lane 3) and splicing complex formation (Figure 2B, lane 3). When PP1γ was pre-incubated with microcystin-LR, spliceosome formation is detected but catalysis is severely reduced (Figure 2A and B, lanes 4). This confirms that splicing inhibition is a specific effect of the phosphatase.

In summary, the data show that Ser/Thr protein dephosphorylation events block pre-mRNA splicing *in vitro* by specifically preventing spliceosome assembly.

PP1 does not affect ATP levels

ATP is required for spliceosome assembly and pre-mRNA splicing. To clarify that the inhibitory effects of protein phosphatases and phosphatase inhibitors do not arise indirectly through depletion of ATP from the extract, we analysed the levels of [α-^{32}P]ATP in splicing extracts by TLC (Figure 3). ATP levels are stable during a 1 h incubation under standard splicing conditions (Figure 3, lane 6) and in extracts treated with either PP1γ (Figure 3, lane 7) or microcystin-LR (Figure 3, lane 8) at concentrations that inhibit splicing. In contrast, when alkaline phosphatase was added to the splicing assay, all the labelled ATP was hydrolysed within 1 h (Figure 3, lane 9) and ~50% in the first 10 min (Figure 3, lane 10). Lanes 2–4 correspond to HeLa splicing extracts incubated without creatine phosphate (CP). These conditions do not support splicing and the data show that >50% of the ATP

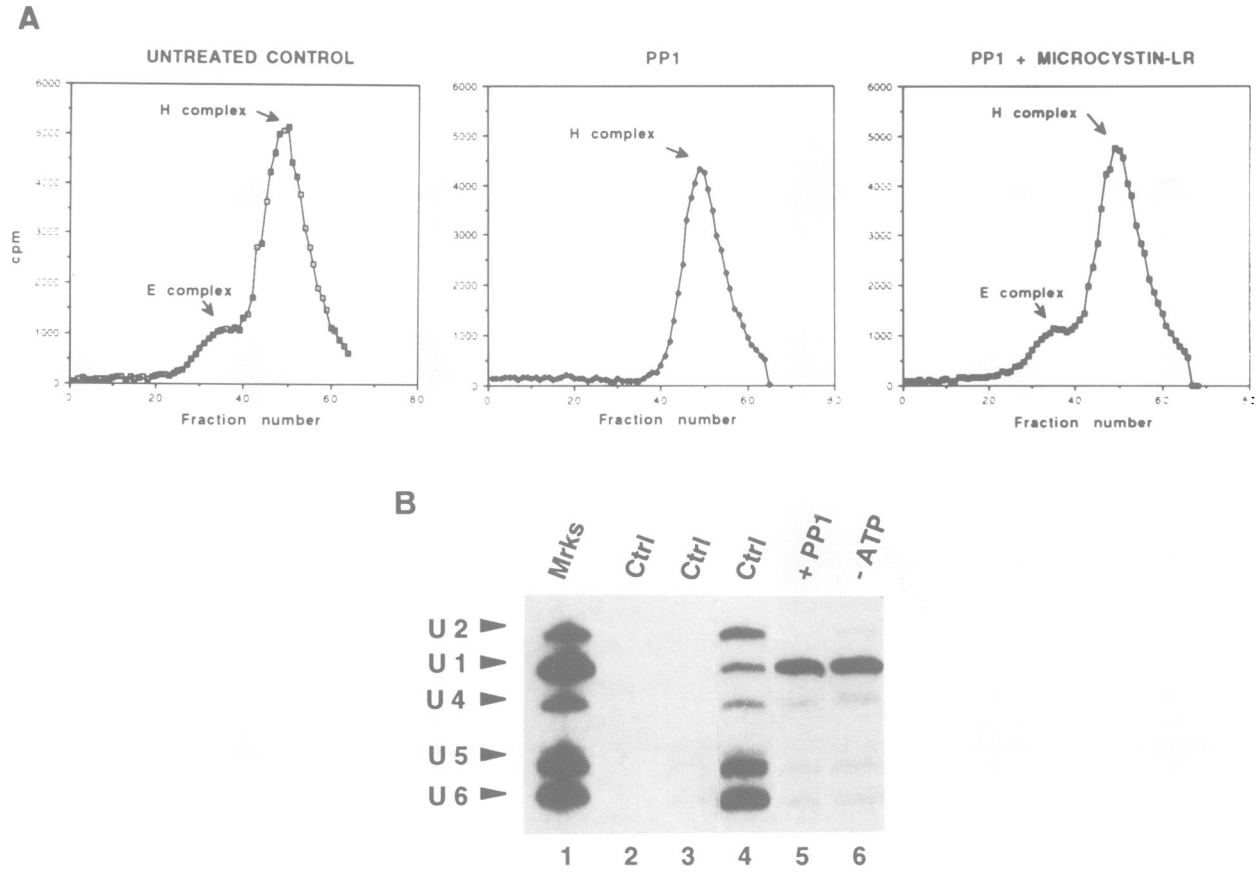

Fig. 4. An early step of splicing complex formation is affected by PP1 treatment. (**A**) In the presence of PP1, no pre-splicing E complex can be detected by gel filtration. Splicing reactions in the absence (untreated control) or presence of protein phosphatase (PP1; 0.1 U/μl) or in the presence of pre-mixed phosphatase with phosphatase inhibitor (PP1 + microcystin-LR; 0.1 U/μl + 1 μM) were fractionated by gel filtration (see Materials and methods). The peaks containing E and H complexes are indicated. (**B**) Only U1 snRNP forms a stable complex with pre-mRNA after PP1γ treatment. Affinity selection of snRNAs from splicing complexes assembled in the absence (lanes 2–4 and 6) or presence (lane 5) of PP1γ (0.6 U/μl). Nuclear extract was pre-incubated at 30°C for 10 min to hydrolyse the endogenous ATP. Splicing reactions were carried out for 40 min. The affinity selection was performed using biotinylated antisense 2′-*O*-Me RNA oligonucleotides targeted to the Ad1 pre-mRNA (Ryder *et al.*, 1990). Affinity-selected RNAs were separated on a 10% denaturing polyacrylamide gel and analysed by Northern hybidization using probes complementary to each of the five spliceosomal snRNAs. Marker lane corresponds to unselected total HeLa nuclear RNA (lane 1). Lane 2 corresponds to incubation of pre-mRNA in splicing reactions without an antisense oligonucleotide, lane 3 shows the level of RNA selected in the absence of precursor, and lanes 4–6 correspond to reactions with both pre-mRNA and oligonucleotide present. In sample 6 no ATP/CP was added to the reaction.

is hydrolysed within 2 min (Figure 3, lane 2) and all of it within 20 min (Figure 3, lane 3). Additional experiments show that exogenously added creatine phosphokinase does not restore splicing or splicing complex formation to PP1-blocked extracts (data not shown). Increasing ATP concentration in the assay also failed to restore splicing to a PP1-treated extract (data not shown).

An early step of splicing complex formation is blocked in PP1-treated extracts

As judged by native gel electrophoresis, no pre-splicing A complex (which contains U1 and U2 snRNPs) can be detected in PP1-treated extracts (Figure 1B; see lanes 1 and 2). Therefore, we addressed whether the pre-spliceosome E (early) complex (Reed, 1990; Michaud and Reed, 1991, 1993), which forms before A complex, could be detected after PP1 treatment. E complex can be assayed by gel filtration as an additional peak eluting prior to hnRNP containing H complexes. We observed E complex in both the untreated control extract and in a control reaction where PP1γ was inhibited by microcystin-LR (Figure 4A). In contrast, only H complexes form in PP1γ-treated extracts (Figure 4A).

Splicing extracts treated with PP1γ were analysed further to determine whether splicing snRNPs could still bind stably to pre-mRNA (Figure 4B). SnRNP binding to pre-mRNA in either a control extract, or in extracts treated with PP1γ, was studied by affinity selection using a biotinylated anti-pre-mRNA 2′-*O*-alkyl oligoribonucleotide (Ryder *et al.*, 1990). Each of the U1, U2, U4, U5 and U6 snRNAs are detected in splicing complexes formed within 40 min in the control extract (Figure 4B, lane 4). The specificity of binding is confirmed by control experiments carried out in the absence of either antisense probe (Figure 4B, lane 2) or in the absence of pre-mRNA (Figure 4B, lane 3). When splicing complex assembly was inhibited by treatment with PP1γ, U1 snRNP showed a higher level of stable complex formation with the pre-mRNA (Figure 4B, lane 5), while the levels of the other four spliceosomal snRNAs were specifically reduced, relative to the untreated control extract (Figure 4B, lane 4). A similar pattern is observed in a control assay where ATP was omitted (Figure 4B, lane 6). Additional analysis showed that the unselected snRNAs were not degraded (data not shown).

We conclude that the inhibitory effect of PP1γ on

spliceosome assembly does not prevent stable binding of U1 snRNP to pre-mRNA, but impairs stable binding of U2 and U4/U6.U5 snRNPs. Interestingly, the inhibition of E complex formation, but not of U1 snRNP binding, seen after PP1γ treatment suggests that there may be an intermediate complex formed between the previously described H and E complexes. A detailed description of this complex requires further investigation.

Restoration of splicing in PP1-inhibited extract

Having established that PP1γ treatment inhibits an early step in the spliceosome assembly pathway, we next sought to identify conditions that could restore splicing activity to inhibited extracts. Splicing was assayed either in the absence of protein phosphatase (Figure 5, lanes 1–5) or in the presence of PP1γ (Figure 5, lanes 6–9). Addition of untreated nuclear extract to the PP1γ-blocked extract restored splicing (Figure 5, see lanes 6 and 7) when added in low amounts which alone could not support splicing (Figure 5, lane 5). To address whether an RNA moiety was important for complementation, nuclear extract was pre-treated with micrococcal nuclease (MCN). The MCN-treated extract showed no splicing activity (Figure 5, lane 3), while a control extract treated with MCN under conditions where the nuclease is inactive does splice the pre-mRNA (Figure 5, lane 2). Addition of MCN extract, or MCN control extract, to the PP1γ-blocked extract restored splicing (Figure 5, lanes 8 and 9). The MCN control extract restored splicing even when used in an amount that was too low to splice on its own (Figure 5, lane 4). We conclude that one or more MCN-resistant factors in nuclear extract can activate splicing in PP1-treated extracts.

SR104 proteins restore splicing in PP1-inhibited extract

Since SR104 splicing factors are known to be phosphoproteins that have essential functions early in splicing complex assembly, we tested whether their addition to PP1γ-treated extracts would restore splicing. SR104 proteins were prepared from HeLa cells as described by Zahler *et al.* (1992), their purity confirmed by SDS−PAGE analysis and their function demonstrated by complementation of splicing in a HeLa S100 extract (data not shown). Addition of purified SR104 proteins to a PP1γ-inhibited splicing extract restored spliceosome assembly (Figure 6B, see lanes 3 and 4) and mRNA formation (Figure 6A, see lanes 3 and 4) to a level comparable with the untreated control (Figure 6A and B, see lanes 4 and 1). The level of splicing/splicing inhibition could be manipulated by varying the concentration of exogenous SR104 proteins and PP1γ, respectively. Thus, increasing the amount of PP1γ requires a corresponding increase in the amount of SR104 proteins to achieve a comparable level of complementation (data not shown). Furthermore, SR104 proteins enhanced splicing of adeno pre-mRNA and complex formation in untreated HeLa nuclear extract (Figure 6A and B, lanes 2). These conditions of increased splicing activity (Figure 6C, see lane 1 with lanes 2 and 7) are still sensitive to phosphatase treatment (Figure 6C, lanes 3–6). Differences were observed between separate HeLa extract preparations, both in their splicing efficiency and in the level of splicing enhancement obtained upon adding exogenous SR104 proteins. However, in all cases treatment with PP1γ inhibited splicing.

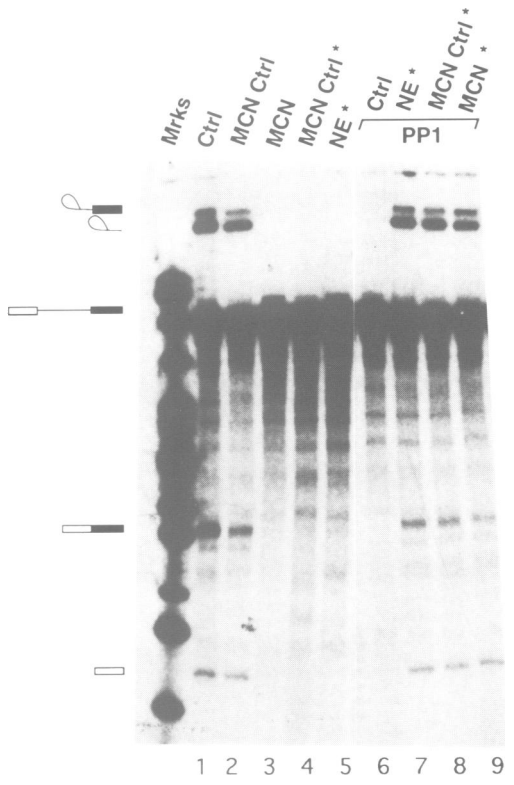

Fig. 5. MCN-treated nuclear extract restores splicing to a PP1γ-blocked extract. Standard splicing assays in the absence (lanes 1–5) or presence (lanes 6–9) of PP1γ. Unless otherwise stated, 7 μl of extract are included in the reactions. Lane 1 corresponds to an untreated control splicing reaction, lane 2 to a MCN control extract (see Materials and methods). Lane 3 shows a standard splicing reaction with MCN-treated extract, lanes 4 and 5 show splicing assays performed using 2.5 μl of MCN control extract and 2 μl of untreated control extract, respectively. PP1γ treatment (0.03 U/μl) shown in lanes 6–9 was performed by pre-incubation of the extract (42 μl/ 3.7 U enzyme) for 10 min on ice. Extracts tested for complementation were added at the beginning of the splicing assay: water (lane 6); 2 μl of standard HeLa nuclear extract (lane 7); 2.5 μl of MCN control extract (lane 8) and 3 μl of MCN-treated extract (lane 9). Low amounts of extract that alone were not sufficient to support splicing are marked with an asterisk.

Phosphorylation state of SR104 proteins is affected by PP1

If endogenous SR104 proteins in HeLa extracts are direct targets for the PP1-mediated inhibition of spliceosome assembly, their phosphorylation state should be altered upon PP1 treatment. To test this, SR104 proteins were detected in HeLa nuclear extracts incubated under splicing conditions using an SR104-specific monoclonal antibody (mAb104; Roth *et al.*, 1990, 1991; Zahler *et al.*, 1992) that recognizes a phosphoepitope (Figure 7). In an immunoblot, the untreated control extract shows the characteristic pattern of SR104 proteins (Figure 7, lane 1; see Zahler *et al.*, 1992). After PP1γ treatment, only a band at ~30 kDa is detected, running with a slightly altered mobility to the doublet of ~30 kDa in the untreated control (Figure 7, see lanes 1 and 2). This pattern was observed at early time points and persisted until the end of the reaction with no evidence of enhanced protein degradation

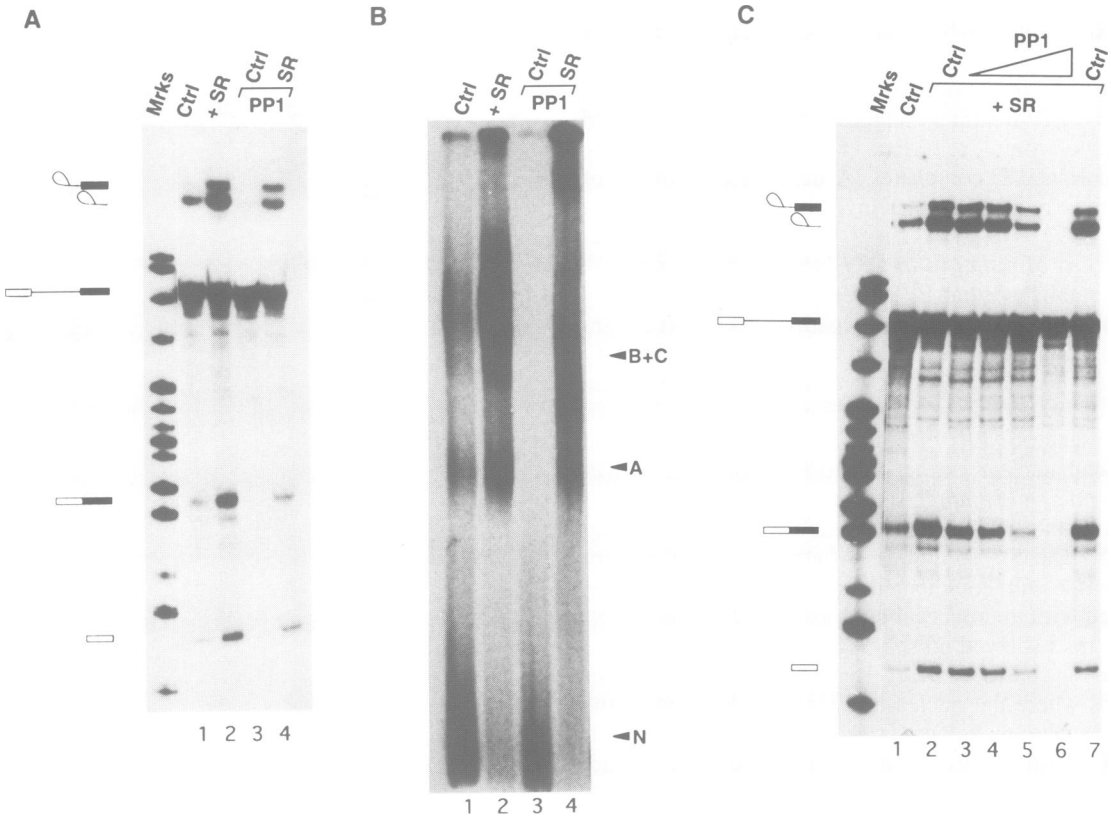

Fig. 6. Purified SR104 proteins restore splicing activity to a PP1γ-blocked extract. The inhibition of splicing complex assembly caused by PP1γ treatment was relieved by the addition of purified HeLa SR104 proteins in a concentration-dependent manner. (**A**) and (**B**) Splicing reactions carried out in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of PP1γ (0.03 U/μl). One microgram of purified SR 104 proteins/20 μl reaction was added at the start of the assay in the case of samples 2 and 4. (**C**) Standard splicing in an untreated control extract (lane 1) and in the presence of exogenously added SR104 proteins (1 μg SR104/20 μl, lanes 2–7). An increasing amount of PP1γ was added to the SR104-treated extracts: 0.0125 U/μl (lane 3), 0.025 U/μl (lane 4), 0.05 U/μl (lane 5) and 0.1 U/μl (lane 6). (A) and (C) Analysis of splicing products by denaturing gel electrophoresis, cartoons indicating the identity of splicing intermediates and products. (B) Analysis of splicing complexes by native gel electrophoresis.

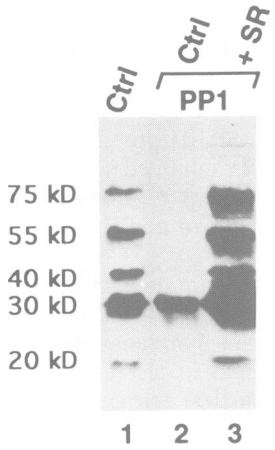

Fig. 7. Endogenous SR104 proteins in HeLa nuclear extract are dephosphorylated upon PP1γ treatment. The phosphorylation state of SR104 proteins was analysed by probing Western blots with mAb104. An untreated splicing reaction control (lane 1) is compared with reactions containing extracts that were treated with PP1γ (0.03 U/μl; lanes 2 and 3) and, in the case of sample 3, where splicing was restored by addition of exogenous HeLa SR104 proteins (1.1 μg SR104/20 μl reaction). Phosphatase treatment was performed by pre-incubation of the extract with PP1γ for 10 min on ice. Purified SR104 proteins were added after 5 min of incubation under standard splicing conditions. All samples shown were incubated for 70 min at 30°C.

(data not shown). In contrast, mAb104 could detect all the SR104 proteins in control extracts without exogenous PP1 throughout the splicing reaction (data not shown). Parallel analysis demonstrated that the pre-mRNA was processed in the control extract, but not in the PP1γ-treated extract (data not shown). The usual pattern of SR104 proteins revealed by mAb104 is detected again when splicing is restored to a PP1γ-treated extract by addition of exogenous SR104 proteins (Figure 7, lane 3). Similarly, additional experiments showed that PP1γ also dephosphorylates purified SR104 proteins (Mermoud, 1994; and data not shown). We conclude that PP1γ treatment removes the mAb104 phosphoepitope from most SR104 proteins and that this correlates with the inhibition of spliceosome assembly. There may also be changes in phosphorylation at other sites in SR104 proteins which are not recognized by mAb104.

The data are consistent with SR104 proteins being direct targets for regulation mediated by PP1. This predicts that addition of the specific phosphatase inhibitor microcystin-LR to PP1γ-treated extract should, if the extract contains the appropriate kinase activities: (i) change the phosphorylation state of the endogenous SR104 proteins and (ii) restore spliceosome assembly to the PP1γ-treated extract. Parallel Western blot and native gel analyses confirm this to be the case (Figure 8). Thus, PP1γ treatment

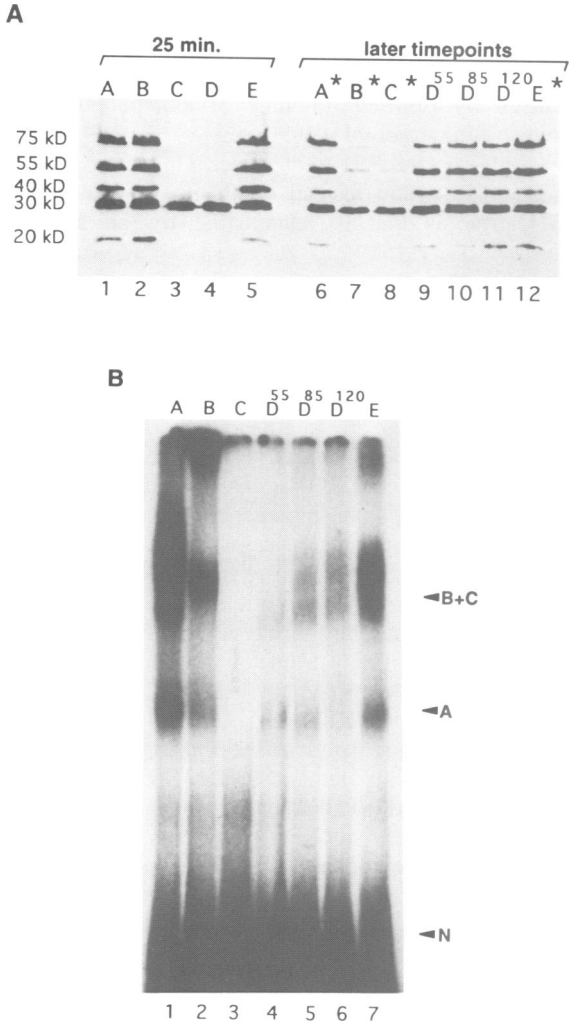

Fig. 8. Addition of microcystin-LR to PP1γ-treated extract results in rephosphorylation of SR104 proteins and restoration of spliceosome assembly. (**A**) Detection of endogenous SR proteins by probing Western blots with mAb104. Samples were analysed after either 25 min (lanes 1–5), 55 min (lane 9), 85 min (lanes 6–8, 10, 12) or 120 min (lane 11) after starting the splicing assay. An untreated splicing control (lanes 1 and 6) was compared with a sample where PP1γ was added either after 25 min of incubation (lanes 2 and 7); or from the beginning of the reaction (lanes 3, 4, 8–11), or was pre-mixed with microcystin-LR and then added at the beginning of the reaction (lanes 5 and 12). We also tested whether SR proteins become rephosphorylated in extracts treated with PP1γ if the phosphatase is subsequently inactivated (samples marked 'D'). In this case, the extract was incubated for 25 min with PP1γ (lane 4), then microcystin-LR was added to the extract to inhibit the phosphatase and the samples analysed at the times shown (lanes 9–11). (**B**) Native gel analysis of splicing complexes formed in samples treated with PP1γ and microcystin-LR as described in (A). The labels at the top of each lane indicate the corresponding samples analysed in (A) and (B). Samples were analysed after either 25 and 45 min (lanes 1–3, 7 and other data not shown), 55 min (lane 4), 85 min (lane 5) or 120 min (lane 6) incubation at 30°C.

removes phosphoepitopes recognized by mAb104 (Figure 8A, lanes 3, 4, 7 and 8) and inhibits splicing complex formation (Figure 8B, lane 3). Subsequent addition of microcystin-LR restores the phosphoepitope (Figure 8A, lanes 9–11) and spliceosome formation (Figure 8B, lanes 4–6). These data also demonstrate that the HeLa nuclear splicing extracts contain kinase activity(ies) that can phosphorylate the SR104 proteins at the epitope recognized

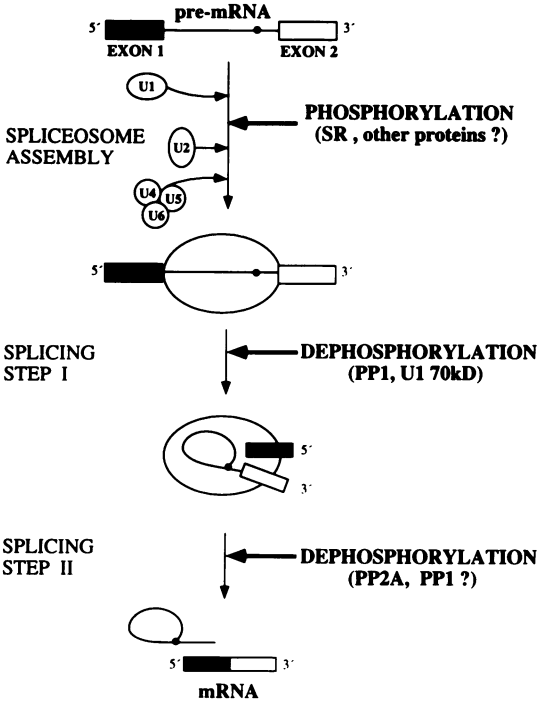

Fig. 9. The cartoon illustrates a model showing distinct steps where splicing may be regulated by a protein phosphorylation mechanism. Phosphorylation is required for early steps in spliceosome assembly (this study). Possible targets for this effect include SR104 protein splicing factors. Once a spliceosome containing U1, U2, U4/U6.U5 snRNPs is assembled, at least two separate dephosphorylation events are required for catalysis, with PP1 needed for the first catalytic step and PP2A (and possibly also PP1) for the second catalytic step (Mermoud *et al.*, 1992; Tazi *et al.*, 1992). Dephosphorylation of the U1 70 kDa protein may be required for the first catalytic step since its thiophosphorylation blocks splicing, but not spliceosome assembly (Tazi *et al.*, 1993).

by mAb104. Addition of PP1γ 25 min after starting the assay does not block spliceosome assembly (Figure 8B, lane 2) or catalysis (see Figure 1), but removes the phosphoepitope recognized by mAb104 (Figure 8A, see lanes 2 and 7).

Discussion

In this study, we have shown that treatment of HeLa nuclear splicing extracts with either human PP1 expressed in *E.coli*, or PP1 purified from rabbit skeletal muscle, selectively inhibits an early step during spliceosome assembly. After PP1 treatment, no pre-splicing E complex can be detected and while U1 snRNP can still form a stable complex with the pre-mRNA, stable binding of U2 and U4/U6.U5 snRNPs is prevented. This contrasts with the effect of adding specific phosphatase inhibitors to the extract, which block splicing catalysis but allow the formation of inactive spliceosomes containing U1, U2 and U4/U6.U5 snRNPs (Mermoud *et al.*, 1992; Tazi *et al.*, 1992). Therefore, as illustrated in Figure 9, protein phosphorylation can affect pre-mRNA splicing either positively or negatively by influencing separate steps in the splicing pathway. These findings are reminiscent of the complex phosphorylation mechanisms that have been shown to regulate many biological processes, including metabolism and the cell division cycle.

Inhibition of splicing by PP1 is a clear effect of protein phosphatase activity since (i) it is sensitive to the specific phosphatase inhibitor microcystin-LR and (ii) inhibition was observed with both purified rabbit muscle PP1 and human PP1γ purified to homogeneity from a bacterial expression system that is free of contaminating eukaryotic proteins. That PP1 does not block catalysis when added after spliceosome assembly underlines the specificity of inhibition. This specificity is further supported by studies on alternative splicing substrates with competing 5′ splice sites (Cardinali *et al.*, 1994). In conditions where both 5′ splice sites are active, PP1γ treatment can block splicing to the upstream 5′ splice site without inhibiting splicing to the downstream 5′ splice site.

A major question that emerges is how protein phosphatases can affect the activity of specific components of the splicing machinery. The catalytic subunits of protein phosphatases are known to have inherent substrate specificities (Cohen, 1989). However, studies on cytoplasmic and nuclear forms of PP1 and PP2A have shown that additional regulation of substrate specificity can be conferred upon native enzymes through the presence of targeting subunits (Cohen, 1989; Bollen *et al.*, 1993; Hubbard and Cohen, 1993). It is possible, therefore, that the inhibition of spliceosome assembly is enhanced by the interaction of exogenous PP1 with free targeting subunits in the HeLa nuclear extract. PP1 activity is present in the HeLa nuclear splicing extracts (Mermoud *et al.*, 1992). It will be important now to isolate native PP1 from the HeLa nuclear extract and to characterize any potential regulatory subunits that can enhance its activity towards components of the splicing apparatus. Another important task is to establish whether PP1 itself, or another protein phosphatase with overlapping substrate specificity, acts upon splicing factors *in vivo*.

Which protein splicing factors may be the key targets whose activity is modulated by PP1? Leaving aside as yet unidentified splicing factors, there are several candidate phosphoproteins whose activities are required at early steps during spliceosome assembly. These include the SR family of non-snRNP protein splicing factors, the U1 snRNP-specific 70 kDa protein and both subunits of the non-snRNP splicing factor U2AF (i.e. $U2AF^{65}$ and $U2AF^{35}$). Although it is important to recognize that when dealing with a crude system, such as the HeLa splicing extract, it is difficult to exclude indirect effects, we have presented here several lines of evidence indicating that one or more of the SR104 proteins may be direct targets whose activity is affected by PP1. Not only are they required at an early stage of spliceosome assembly (Krainer *et al.*, 1990, 1991; Ge *et al.*, 1991; Fu and Maniatis, 1992a,b; Zahler *et al.*, 1992, 1993; Fu, 1993), which correlates with the stage at which PP1 inhibits, addition of purified SR104 proteins is sufficient to restore spliceosome assembly in a PP1-treated extract. SR104 proteins are reported to be reversibly phosphorylated *in vivo* (Roth *et al.*, 1991; Gui *et al.*, 1994). We show in this study that the phosphorylation state of endogenous SR104 proteins in HeLa nuclear extracts changes when splicing is inhibited by PP1. An interesting comparison can be drawn between the PP1-treated HeLa nuclear extract and HeLa S100 extracts. In both cases, splicing and spliceosome assembly do not take place and the assembly block appears to occur at a similar early step. Both extracts can be activated by addition of exogenous, purified SR104 proteins (this study; Zahler *et al.*, 1992). In S100 extracts, the splicing defect is caused by limiting amounts of endogenous SR104 proteins (Krainer *et al.*, 1990; Fu *et al.*, 1992; Zahler *et al.*, 1993). In the PP1-treated HeLa nuclear extract, we propose that it may instead be the loss of function of SR104 proteins that is responsible for an analogous inhibition.

The phosphorylation state of at least some proteins in the HeLa splicing extract is dynamic, since phosphate groups are continuously added and removed by competing kinase and phosphatase activities. As a consequence, the steady-state level of protein phosphorylation will change rapidly in response to any treatment that affects the relative level of these competing activities. We show here that PP1 can dephosphorylate SR104 proteins. Recently, several studies have reported the characterization of kinase activities that phosphorylate proteins with SR domains (Woppmann *et al.*, 1993; Gui *et al.*, 1994). One activity is associated with snRNP particles and phosphorylates serine residues in the serine–arginine repeat motif (Woppmann *et al.*, 1993). This activity phosphorylates the U1 snRNP 70 kDa protein at a subset of the sites of phosphorylation detected *in vivo*. Gui *et al.* (1994) identified a cell cycle-regulated serine kinase that can phosphorylate SR splicing factors, SRPK1 (SR protein specific kinase 1). Purified SRPK1 can induce disassembly of speckled intranuclear snRNP structures in interphase nuclei. There is also evidence that the phosphorylation state of SR104 proteins is dynamic *in vivo*, since they were reported to be hyperphosphorylated in metaphase cells (Roth *et al.*, 1990; Gui *et al.*, 1994).

Apart from SR104 proteins, the other major candidates for regulated target proteins are U2AF and the U1 70 kDa protein, which both have an SR domain and are required at an early stage of spliceosome assembly (Ruby and Abelson, 1988; Séraphin and Rosbash, 1989; Barabino *et al.*, 1990; Zamore *et al.*, 1992; Zhang *et al.*, 1992). Although we have no evidence at present whether U2AF or U1 70 kDa is affected, either directly or indirectly, by treatment of HeLa nuclear extracts with PP1, it is possible that their activity could be regulated by phosphorylation mechanisms. In the case of U1 snRNP 70 kDa protein, Tazi *et al.* (1993) have shown that its thiophosphorylation inhibits splicing *in vitro*, but not spliceosome assembly. This is consistent with the dephosphorylation of U1 70 kDa protein being required for catalysis after spliceosome formation has occurred (see Figure 9). It is not yet known whether the phosphorylation state of U1 70 kDa might also influence spliceosome assembly.

The E complex, i.e. the earliest functional mammalian pre-splicing complex detected, contains U1 snRNP, both subunits of U2AF and at least five additional spliceosome-associated proteins. In E complex the 5′ and 3′ splice sites are functionally associated (Michaud and Reed, 1991, 1993). That no E complex forms in PP1-treated nuclear extracts suggests that either stable binding of factors to the respective 5′ and 3′ splice sites, or stable interactions between these components, are prevented by dephosphorylation. Recently, SC35 and ASF/SF2 were found to interact specifically with both the U1 70 kDa protein and with the small subunit of U2AF (Wu and Maniatis, 1993).

Therefore it was proposed that SR proteins play a role in promoting or stabilizing interactions between the separate ends of the intron, e.g. the U1 and U2 snRNP, at an early stage of spliceosome assembly. These 'bridging' functions may be affected by changes in phosphorylation mediated by PP1. The complex we detect accumulating after PP1 treatment may correspond to an otherwise transient intermediate between the H and E complexes which has not been previously reported. Therefore, an important task will be to characterize in detail this arrested complex, and determine its composition and its relationship to previously described complexes that form at early stages of spliceosome assembly (Legrain *et al.*, 1988; Séraphin and Rosbash, 1989; Michaud and Reed, 1991; Jamison *et al.*, 1992; Fu, 1993). It will also be important to relate the present *in vitro* findings with possible *in vivo* roles of reversible phosphorylation in regulating pre-mRNA splicing. In this regard it is interesting that one essential pre-mRNA splicing factor in *Schizosaccharomyces pombe*, prp4$^+$, codes for a predicted Ser/Thr kinase (Alahari *et al.*, 1993). Based on the network of phosphorylation mechanisms involved in regulating a wide range of biological processes, we may anticipate that complex effects of phosphorylation on pre-mRNA splicing remain to be uncovered.

Materials and methods

Protein phosphatases and phosphatase inhibitors

Human PP1γ was expressed in *E.coli* and purified as described by Alessi *et al.* (1993). The isolation and sequence of the PP1γ clone was described by Barker *et al.* (1993). The catalytic subunits of PP1 and PP2A (Cohen *et al.*, 1988) were purified to homogeneity from rabbit skeletal muscle and kindly provided by Prof. Philip Cohen (University of Dundee). One unit of activity catalyses the dephosphorylation of 1 nmol of glycogen phosphorylase in 1 min. Microcystin-LR was purchased from Sigma.

In vitro splicing assays

HeLa cell nuclear extracts were prepared as described by Mermoud *et al.* (1992). Splicing assays were performed using uniformly labelled, capped pre-mRNAs incubated with nuclear extracts using the *in vitro* splicing conditions described by Lamond *et al.* (1987). Adenovirus major late precursor (adeno pre-mRNA) was transcribed from *Sau*3A-digested plasmid pBSAd1 (Konarska and Sharp, 1987). Splicing products were separated on 10% polyacrylamide/8 M urea denaturing gels, run in 1× TBE. Splicing complexes were separated on non-denaturing agarose–polyacrylamide composite gels, as described by Lamond *et al.* (1989).

Micrococcal nuclease treatment of nuclear extracts

MCN was purchased from Sigma and further purified by FPLC chromatography on MonoS, following the protocol of Young *et al.* (1991). MCN digestion was performed essentially as described by Krainer and Maniatis (1985). Sixty microlitres of HeLa nuclear extract were mixed with $CaCl_2$ to give a final concentration of 1 mM and then MCN added. After incubation for 25 min at 30°C, the nuclease was inactivated by adding EGTA to a final concentration of 1 mM. The MCN control extract (MCN Ctrl) corresponds to an otherwise identical sample where EGTA was present from the beginning of the incubation at 30°C. Aliquots of the MCN and MCN Ctrl extracts were deproteinized and analysed by Northern blotting to ensure that snRNAs were degraded in the MCN extract, but not in the MCN control.

Streptavidin agarose affinity selection assay and Northern hybridization analyses

Antisense affinity selection assays and Northern hybridization analyses were performed as described by Ryder *et al.* (1990). The sequence of the biotinylated, anti-adeno intron 2′-*O*-Me RNA oligo was as follows: 5′-IACCAIAUIIACICIICC-3′ (I = inosine) with four additional biotinylated deoxycytidines at the 5′ terminus (Sproat *et al.*, 1989).

Gel filtration of pre-splicing E complexes

Splicing reactions (300 μl) were set up with RNasin and dithiothreitol (DTT), but without the addition of exogenous ATP, magnesium and CP. After pre-incubation in the absence or presence of protein phosphatase for 5 min at room temperature, pre-mRNA was added and reactions were incubated for 14 min at 30°C, and subsequently loaded directly onto a 1.5×50 cm Sephacryl S-500 column equilibrated in FPS buffer [20 mM Tris (pH 7.8), 0.1% Triton X-100, 60 mM KCl, 2.5 mM EDTA]. Fractionation by gel filtration was performed as described by Reed (1990); the column flow rate was increased to 0.15 ml/min..

TLC

Aliquots (1/20th of each sample) of standard splicing reactions containing α-^{32}P-labelled ATP were separated by TLC on Polygram PEI cellulose MN 300 paper, developed using 300 mM aqueous trisodium citrate at pH 7.0. Standards of unlabelled ATP and ADP were located, after drying, under a 254 nm UV lamp.

SDS–PAGE and immunoblotting

For Western blots, 40 μl splicing reactions containing 14 μl of nuclear extract (9 mg/ml) were used. Reactions were stopped by the addition of Laemmli sample buffer and proteins were separated on 12.5% SDS–polyacrylamide gels using the method of Lehmeier *et al.* (1990). After transfer of proteins on nitrocellulose membranes using semi-dry electrotransfer, Western blot analysis was performed as described by Blencowe *et al.* (1993). The immunoblots shown were developed using ECL chemiluminescence (Amersham, UK), in combination with horseradish peroxidase secondary antibodies, as per the manufacturer's instructions.

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