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# Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing

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## ABSTRACT

**We have used a combination of highly specific protein phosphatase inhibitors and purified mammalian protein phosphatases to show that at least two separate Ser/Thr protein phosphatase activities are required for pre-mRNA splicing, but not for spliceosome assembly. Okadaic acid, tautomycin, and microcystin-LR, which are potent and specific inhibitors of PP1 and PP2A, two of the four major types of Ser/Thr-specific phosphatase catalytic subunits, block both catalytic steps of the pre-mRNA splicing mechanism in HeLa nuclear extracts. Inhibition of PP2A inhibits the second step of splicing predominantly while inhibition of both PP1 and PP2A blocks both steps, indicating a differential contribution of PP1 and PP2A activities to the two separate catalytic steps of splicing. Splicing activity is restored to toxin-inhibited extracts by the addition of highly purified mammalian PP1 or PP2A. Protein phosphatase activity was not required for efficient assembly of splicing complexes containing each of the U1, U2, U4/U6 and U5 snRNPs. The data indicate that reversible protein phosphorylation may play an important role in regulating the pre-mRNA splicing mechanism.**

## INTRODUCTION

Reversible phosphorylation of proteins is one of the major mechanisms used to regulate cellular processes, including metabolism, initiation of mitosis and progression through the cell cycle, macromolecular synthesis and breakdown, transcription activation and translation. Protein phosphorylation can act either to stimulate or to inhibit cellular processes, since some substrates are activated by addition of phosphate while others are inhibited (1). Proteins can be phosphorylated on either serine, threonine or tyrosine residues. However, in mammalian cells over 99% of protein phosphorylation takes place on serine and threonine residues, and protein phosphatases (PP) which dephosphorylate these residues are classified as forms of PP1, PP2A, PP2B or PP2C, according to which catalytic subunit is present in the native enzyme (2, 3). In most cases, the native forms of Ser/Thr-specific protein phosphatases are comprised of one of the catalytic subunits together with additional proteins that modulate their activity and/or substrate specificity and intracellular localisation (2, 3). All known Ser/Thr-specific phosphatases are sensitive to inhibition by fluoride, pyrophosphate and EDTA (4). In addition, protein phosphatases containing the PP1 or PP2A catalytic subunit can be selectively inhibited by specific toxins such as the polyketal fatty acids okadaic acid and tautomycin and the cyclic heptapeptide microcystin-LR (5, 6, 7). The great specificity of these toxins has recently led to their widespread use for identifying biological processes that are regulated by forms of PP1 or PP2A enzymes (8, 9).

In this study we have investigated whether the splicing of mammalian pre-mRNA substrates is also subject to regulation by reversible protein phosphorylation. The splicing of specific pre-mRNAs can be studied *in vitro* using extracts prepared from HeLa cells (10, 11, 12). Previous analyses have established that splicing takes place by a two-step mechanism (13, 14) which likely involves two sequential transesterification reactions (15). The first step involves cleavage of the pre-mRNA at the 5′ intron–exon junction, generating reaction intermediates corresponding to the free 5′ exon and a 'lariat' intron which is still joined to the 3′ exon. The 'lariat' structure results from the formation of a 2′–5′ phosphodiester bond linking the 5′ end of the intron to a 2′-OH group of an adenosine residue at an intron sequence close to the 3′ splice site. The second step results in ligation of the exon sequences to yield mRNA and release of the intron, still in the 'lariat' configuration. Both these catalytic reactions take place within a dedicated complex, termed a spliceosome, and are preceded by a series of assembly steps. The subunits of the splicing apparatus, specifically the U1, U2, U4/U6 and U5 snRNPs, together with other non-snRNP protein splicing factors, associate with the pre-mRNA in an ordered pathway to form a functional spliceosome (16, 17). The splicing reaction *in vitro* requires magnesium and ATP, both of which are needed at multiple stages, including the assembly and catalysis steps.

Here, we demonstrate that Ser/Thr-specific protein phosphorylation can regulate the pre-mRNA splicing mechanism *in vitro*. In particular, we show that forms of PP1 and PP2A are required for both catalytic steps of splicing but not for spliceosome assembly.

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## MATERIALS AND METHODS

The catalytic subunits of PP1 and PP2A (18) and inhibitor-2 (19) were purified to homogeneity from rabbit skeletal muscle. Microcystin-LR was provided by Prof. G.Codd (Dept. of Biological Sciences, University of Dundee), tautomycin by Dr. K.Isono (Antibiotics Laboratory, Institute of Physical and Chemical Research, Saitona, Japan) and okadaic acid by Dr. Y.Tsukitani (Fujisawa Pharmaceutical Company, Tokyo, Japan). All toxins were diluted in aqueous buffer before use. Restriction enzymes were purchased from New England Biolabs and RNAsin from Promega. T3 and T7 RNA polymerases were purchased from Stratagene. $\alpha$-($^{32}$P)-UTP and Hybond membrane were purchased from Amersham. Streptavidin agarose beads were purchased from Sigma. HeLa cells used to prepare nuclear extracts were purchased from the Computer Cell Culture Centre (Mons, Belgium).

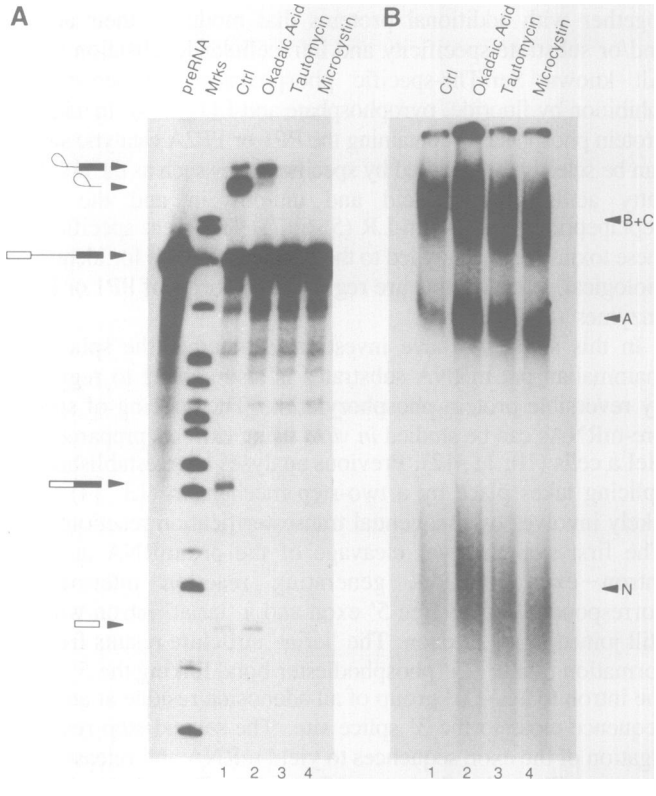


**Figure 1.** Protein phosphatase inhibitors block pre-mRNA splicing. The phosphatase inhibitors okadaic acid, tautomycin and microcystin-LR block pre-mRNA splicing (A) but not the assembly of splicing complexes (B). HeLa nuclear extract was incubated under standard splicing conditions for 120 min. at 30°C (see Materials and Methods). The lane marked 'Ctrl' corresponds to a splicing reaction carried out in the absence of phosphatase inhibitors (lane 1). In lanes 2, 3 and 4 okadaic acid, tautomycin and microcystin-LR were used at a final concentration of 1 $\mu$M, added at the beginning of the splicing assay without preincubation. (A) The splicing of Ad1 pre-mRNA was analysed on a 10% polyacrylamide/8 M urea gel. The structure of the pre-mRNA, splicing intermediates and products are indicated by cartoons with exons drawn as solid boxes and introns as lines. Markers are end-labeled MspI-digested pBR322 fragments ('Mrks') and unspliced pre-mRNA ('preRNA'). (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' stands for non-specific binding.

### HeLa cell nuclear extracts

HeLa cell nuclear extracts were prepared as described previously (20, 21) with the following modification; intact HeLa cells ($5\times10^9$) were harvested by centrifugation and washed once with ice cold PBS. Cells were then pelleted again by centrifugation at 2,000 rpm in an SS34 rotor for 10 minutes at 4°C and resuspended in 5× the packed cell volume (pcv) of buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT). Cells were incubated on ice for 10 minutes, centrifuged as before and resuspended in 2× pcv of buffer S (20 mM Hepes pH 7.9, 10% Glycerol, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT). Cells were lysed in buffer S by 12 strokes with a Dounce homogenizer using an A-type pestle. Additional steps were as described by Barabino *et al.* (21).

### Splicing assays

Splicing assays were done using uniformly labeled, capped pre-mRNAs incubated with nuclear extracts using the *in vitro* splicing conditions described by Lamond *et al.* (22). Adeno pre-mRNA was transcribed from Sau3A-digested plasmid pBSAd1 (23).

Splicing products were separated on 10% polyacrylamide/8 M urea denaturing gels, run in 1×TBE. Splicing and snRNP complexes were separated on non-denaturing agarose-polyacrylamide composite gels, as described by Lamond *et al.* (24).

### 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.* (25).

### Protein phosphatase assays

PP1 and PP2A were assayed by the dephosphorylation of 10 $\mu$M glycogen phosphorylase (18, 26). One Unit of activity is that amount which catalyses the dephosphorylation of 1 $\mu$mole of glycogen phosphorylase in one minute. When inhibitor-2 was included in the assay, diluted nuclear extracts and inhibitors were preincubated for 15 min at 30°C, prior to initiating the reaction by addition of substrate, in order to ensure complete inhibition of PP1.

## RESULTS

### Inhibition of protein phosphatases blocks splicing

The Ser/Thr-specific protein phosphatase inhibitors okadaic acid, tautomycin and microcystin-LR were used to investigate whether protein phosphorylation is involved in the regulation of pre-mRNA splicing in HeLa nuclear extracts (Figure 1). In comparison with the control splicing reaction (Figure 1A, lane 1), all three toxins used at a final concentration of 1 $\mu$M blocked the formation of spliced mRNA and excised intron products from a pre-mRNA derived from the major late transcript of adenovirus (Figure 1A, lanes 2–4). An interesting difference was that okadaic acid caused an accumulation of splicing intermediates while both tautomycin and microcystin-LR blocked the formation of intermediates as well as products (Figure 1A, cf. lane 2 with 3 and 4). However, in the okadaic acid treated extract the total amount of pre-mRNA processed was lower than in the untreated control extract (Figure 1A, cf lanes 1 and 2), indicating that

okadaic acid may also partially inhibit formation of splicing intermediates. We observed identical inhibitory effects of the phosphatase inhibitors on all pre-mRNA substrates tested and with multiple independent preparations of HeLa nuclear extracts (data not shown).

A parallel analysis of splicing complex assembly showed that none of the inhibitors prevented spliceosome formation (Figure 1B). Even where accumulation of splicing intermediates and products was completely inhibited by tautomycin or microcystin-LR, formation of splicing complexes was detected using a native gel assay (Figure 1B, lanes 3 and 4). A similar inhibition of both steps of pre-mRNA splicing, but not spliceosome assembly, was observed in HeLa nuclear extracts treated during their preparation with 600 mM sodium fluoride and 30 mM pyrophosphate, which also potently inhibit Ser/Thr-specific protein phosphatases (data not shown). Additional experiments showed that the rate of splicing complex assembly was not reduced in extracts treated with phosphatase inhibitors (data not shown).

The splicing complexes formed in extracts treated with phosphatase inhibitors were analysed further to determine whether each of the splicing snRNPs could still assemble with pre-mRNA (Figure 2). The snRNP composition of splicing complexes formed in either a control extract, or in extracts treated with microcystin-LR, okadaic acid or tautomycin were analysed by affinity selection using a biotinylated anti-pre-mRNA 2'-O-alkyl oligoribonucleotide as described by Ryder *et al.* (25). Each of the U1, U2, U4, U5 and U6 snRNAs are detected in splicing complexes formed in the absence of phosphatase inhibitors (Figure 2, lane 2). The specificity of the snRNA selection is demonstrated by control experiments done in the absence of either pre-mRNA (Figure 2, lane 1) or in the absence of antisense probe (Figure 2, lane 3). A similar pattern of specific snRNA selection was obtained from an extract where splicing was blocked by preincubation with microcystin-LR (Figure 2, lane 5). Control experiments done with the microcystin-LR-treated extract where either the pre-mRNA (Figure 2, lane 4) or antisense probe (Figure 2, lane 6) were omitted, confirm that the snRNA selection is specific. A similar pattern of snRNA selection was obtained when splicing was inhibited through addition of microcystin-LR without extended preincubation (Figure 2, lane 7). Similar results were also observed with extracts inhibited by either okadaic acid or tautomycin (Figure 2, lanes 8 and 9).

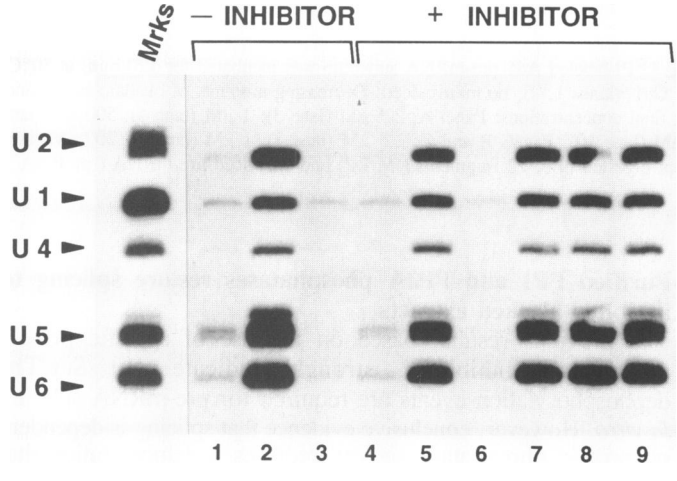


**Figure 2.** snRNA composition of splicing complexes assembled in the presence of phosphatase inhibitors. Affinity selection of snRNAs from splicing complexes assembled in the absence (lanes 1–3) or presence (lanes 4–9) of the phosphatase inhibitors okadaic acid, tautomycin and microcystin-LR. Lanes 4–6 correspond to nuclear extract that was preincubated on ice for 20 min. with 465 nM microcystin-LR before the splicing assay was initiated. All samples, including controls (lanes 1–3), were similarly preincubated on ice and microcystin-LR (lane 7), okadaic acid (lane 8) and tautomycin (lane 9) were added to a final concentration of 1 $\mu$M at the beginning of the splicing assay. Splicing reactions were incubated for 40 min. as described in Materials and Methods. The affinity-selection was performed using biotinylated antisense 2'-OMe RNA oligonucleotides targeted to the Ad1 pre-mRNA (25). Affinity selected RNAs were separated on a 10% denaturing polyacrylamide gel and analyzed by Northern hybridization using probes complementary to each of the 5 spliceosomal snRNAs. Marker lane corresponds to unselected total HeLa nuclear RNA. Lanes 1 and 4 show the level of RNA selected in the absence of precursor, lanes 3 and 6 correspond to incubation of pre-mRNA in splicing reactions without an antisense oligonucleotide, and lanes 2, 5, 7, 8 and 9 correspond to reactions with both pre-mRNA and oligonucleotide present.

We conclude that Ser/Thr-specific protein phosphatase inhibitors block the catalytic steps of splicing but not the assembly of each of the splicing snRNPs with pre-mRNA.

## Distinct protein dephosphorylation steps are required for splicing

In the previous experiments, okadaic acid primarily inhibited the second step of splicing while tautomycin and microcystin-LR blocked both the first and second steps (Figure 1A). Okadaic acid is known to be a less potent inhibitor of PP1 than either tautomycin or microcystin-LR, while all three toxins strongly inhibit PP2A (6, 7, 27). Given these properties, a major role for a form of PP1 at the first catalytic step of splicing would explain why step one was inhibited by tautomycin and microcystin-LR but only weakly by okadaic acid. Since okadaic acid potently inhibited the second step of splicing at the same concentration where the first step was relatively unaffected, the data further indicate a major role for a form of PP2A at step two.

The above model makes two predictions; (a) that okadaic acid should strongly block also the first step of splicing when tested at higher concentrations and (b) that a selective inhibition of the second step of splicing should not occur at lower concentrations of tautomycin or microcystin-LR, since tautomycin inhibits PP1 more strongly than PP2A (6) and microcystin-LR inhibits PP1 and PP2A at similar concentrations (7). To test these predictions the inhibitory effects of all three toxins were assayed over the range of (final) concentrations 2.5 $\mu$M to 10 nM (Figure 3). At higher concentrations okadaic acid indeed blocked both steps of splicing (Figure 3A, lanes 3 and 4). At progressively lower concentrations of okadaic acid increasing levels of splicing intermediates were detected (Figure 3A, lanes 5–9), while at the lowest concentration spliced products were also formed (Figure 3A, lane 10). The absolute concentration of okadaic acid required to inhibit both steps of splicing varied slightly between different extract preparations and was also influenced by preincubation of the toxin with the nuclear extract. However, for all nuclear extracts tested, okadaic acid showed a concentration dependent, differential inhibitory effect on the first and second steps of splicing. Additional experiments also showed that okadaic acid inhibited splicing even when the incubation time of the splicing assay was extended up to three hours (data not shown). This indicates that okadaic acid causes a major block in splicing, rather than a more modest change in the reaction kinetics.

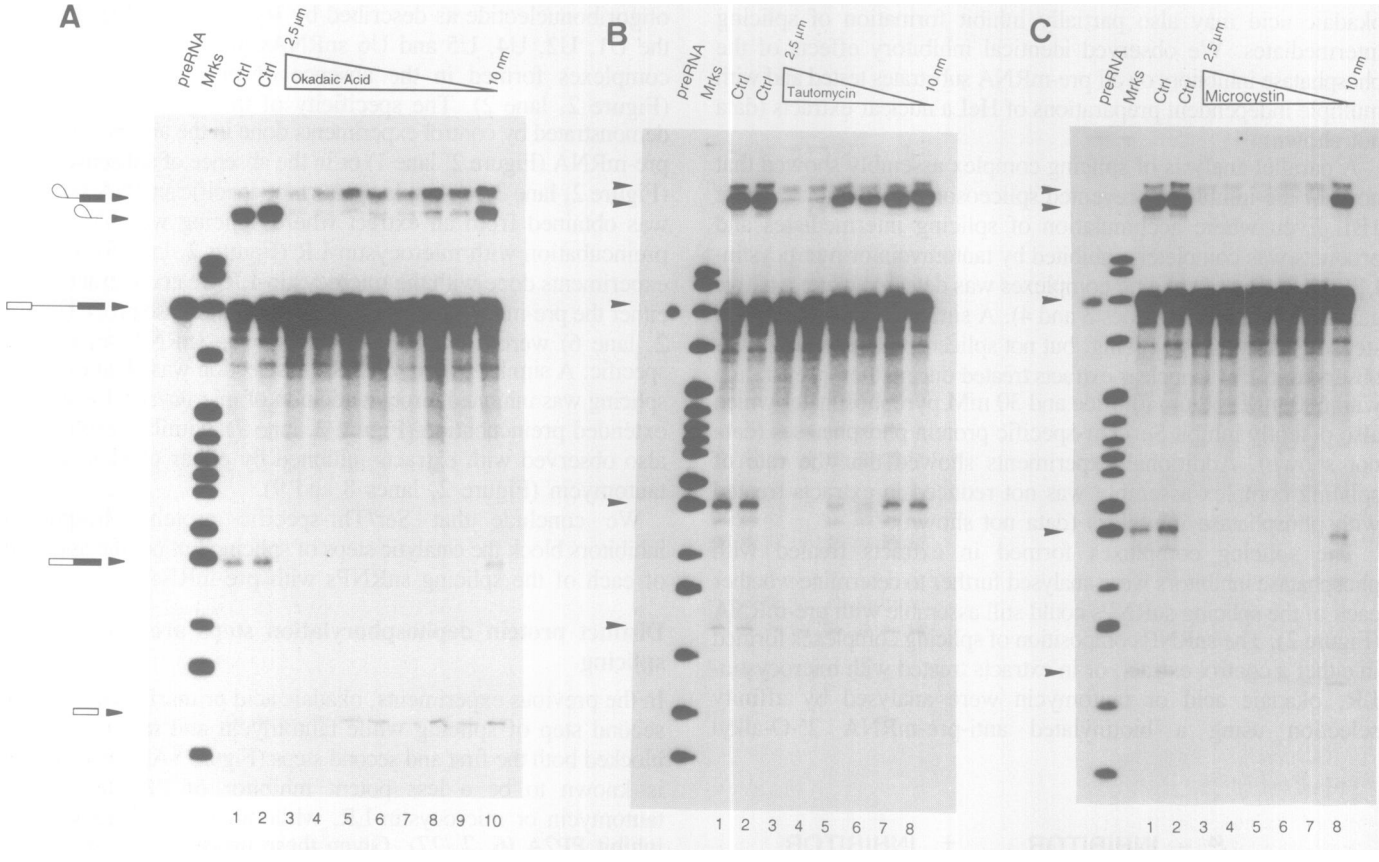


**Figure 3.** Concentration dependence of splicing inhibition by phosphatase inhibitors. [$^{32}$P]-labeled Ad1 pre-mRNA substrate was incubated for 120 min. at 30°C. When present, phosphatase inhibitors were added at the beginning of the reaction: 'Ctrl' (lanes 1, 2), no toxin added. Decreasing amounts of okadaic acid (panel A), tautomycin (panel B) or microcystin-LR (panel C) were added at the following final concentrations: Panel A: 2.5 μM (lane 3); 1 μM (lane 4); 500 nM (lane 5); 300 nM (lane 6); 200 nM (lane 7); 100 nM (lane 8); 50 nM (lane 9) and 10 nM (lane 10). Panels B and C: 2.5 μM (lane 3); 1 μM (lane 4); 200 nM (lane 5); 100 nM (lane 6); 50 nM (lane 7); 10 nM (lane 8). Markers are end-labeled MspI-digested pBR322 fragments ('Mrks') and unspliced pre-mRNA ('preRNA').

At the higher concentrations tested, tautomycin and microcystin each blocked both steps of splicing (Figure 3B and C, lanes 3 and 4). Consistent with their known inhibitory properties, microcystin-LR blocked splicing at lower concentrations than tautomycin (cf. Figure 3B, lanes 5–7 and Figure 3C, lanes 5–7). In contrast with okadaic acid, however, neither drug caused an accumulation of splicing intermediates at lower concentrations but instead allowed both steps of splicing (Figure 3B and C). In extracts treated with low concentrations of inhibitors the mRNA level was slightly lower than in untreated control extracts. The cause of this effect has not yet been investigated. We note that higher concentrations of all the toxins are required to inhibit splicing than have been shown to inhibit PP1 or PP2A in standard assays using purified substrates and highly diluted enzymes. This is due to the high levels of endogenous phosphatases present in the crude nuclear extracts used for *in vitro* splicing assays, as documented below. Since the toxins bind stoichiometrically to PP1 and PP2A, the amounts required to inhibit these enzymes depends upon the total phosphatase concentration in the extract.

In summary, analysis of the concentration dependence for inhibition of pre-mRNA splicing by the separate phosphatase inhibitors under study supports a model in which PP1 and PP2A contribute differentially to distinct dephosphorylation events required for the first and second steps of splicing.

## Purified PP1 and PP2A phosphatases restore splicing to inhibitor-blocked extracts

The previous results, based on the use of specific protein phosphatase inhibitors, strongly indicate that Ser/Thr dephosphorylation events are required for pre-mRNA splicing *in vitro*. However, conclusive evidence that splicing is dependent on protein phosphatase activity requires a demonstration that addition of exogenous protein phosphatase to an inhibitor-blocked extract is sufficient to restore splicing activity. To assay for restoration of splicing activity we have used catalytic subunits of PP1 and PP2A which have been purified to apparent homogeneity from rabbit skeletal muscle (Figure 4).

Extracts were assayed for splicing activity either in the absence of inhibitors (Figure 4, A and B, lanes 1) or in the presence of microcystin-LR (Figure 4A, lanes 2–4) or okadaic acid (Figure 4B, lanes 2–4). As observed previously, both microcystin-LR and okadaic acid potently inhibited splicing (Figure 4, A and B, lanes 2). Addition of highly purified PP1 or PP2A to the microcystin-LR or okadaic acid inhibited extracts restored formation of splicing intermediates and products in each case (Figure 4, A and B, lanes 3 and 4). In additional experiments, the efficiency of splicing restoration was observed to be dependent upon the concentration of phosphatase added and the absolute level of phosphatase required to restore splicing correlated closely

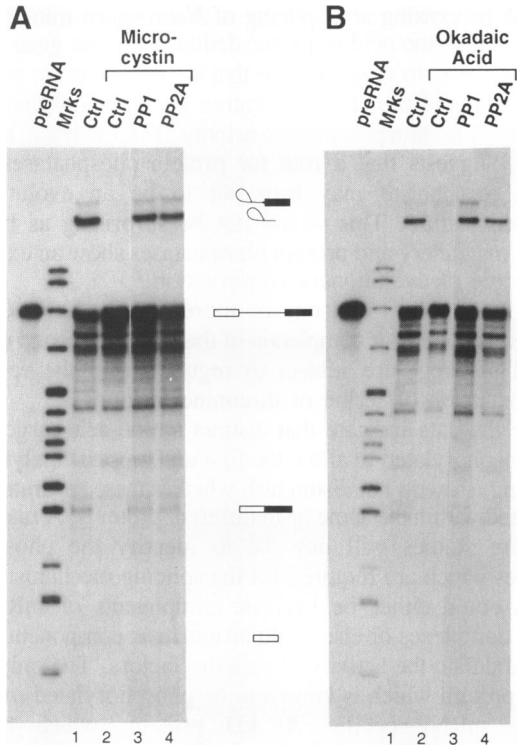


**Figure 4.** Purified phosphatases restore splicing activity to toxin-inhibited extracts. The inhibition of splicing caused by microcystin-LR (A) or by okadaic acid (B) was relieved by the addition of highly purified PP1 or PP2A catalytic subunits. Lane 1 shows a splicing reaction with Ad1 pre-mRNA carried out in the absence of any inhibitor. In panel A, lanes 2–4 correspond to splicing assays performed using an extract that was preincubated on ice with microcystin-LR at a final concentration of 280 nM for 20 min. At the beginning of the splicing assay either $dH_2O$ (lane 2), 3 Units PP1 (lane 3), or 1 Unit PP2A (lane 4) was added. In panel B, lanes 2–4 correspond to splicing assays performed using an extract that was preincubated on ice with okadaic acid at a final concentration of 480 nM for 20 min. At the beginning of the splicing assay either $dH_2O$ (lane 2), 1 Unit PP1 (lane 3), or 2 Units PP2A (lane 4) was added. Splicing products were analyzed on a 10% polyacrylamide/8 M urea gel.

with the concentrations of inhibitors used in different extracts (data not shown). As shown in Figure 4, both PP1 and PP2A can restore splicing activity in inhibited extracts to a level comparable with that seen in the uninhibited control extract (Figure 4, A and B, cf. lanes 1 with lanes 3 and 4).

### Analysis of phosphatase levels in HeLa nuclear splicing extracts

From the present study it is inferred that Ser/Thr-specific protein phosphatases, in particular PP1 and PP2A, are required for pre-mRNA splicing. To establish that endogenous protein phosphatase activities of this type are indeed present in the HeLa nuclear extracts used for *in vitro* splicing experiments, levels of phosphatase activity were measured in such extracts (Table 1). For these assays dephosphorylation of a standard substrate, phosphorylase a, was measured in the presence and absence of phosphatase inhibitors (for details of assay see Materials and Methods). The results show that both PP1 and PP2A activities are present in HeLa nuclear splicing extracts. Consistent with previous studies of protein phosphatase activity in rat nuclear extracts (28), the contribution of PP1 to the phosphorylase

**Table 1.** Levels of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) in HeLa nuclear extracts

| Protein Phosphatase | Microcystin in Extract | PhP Activity mU/ml |
|---|---|---|
| PP1 | – | 9.7 +/– 0.7 |
| PP1 | + | 0 |
| PP2A | – | 4.8 +/– 0.3 |
| PP2A | + | 0 |

Nuclear extracts were assayed at a 150-fold final dilution as described previously (26, 18). PP1 was the phosphorylase phosphatase (PhP) activity inhibited by inhibitor-2 (200 nM) or not inhibited by 2 nM okadaic acid, while PP2A was the activity that was blocked by 2 nM okadaic acid or resistant to 200 nM inhibitor-2. The relative contributions of PP1 and PP2A to the PhP activity assessed by both methods agreed to +/– 5%. The results are given as +/– SEM for three separate experiments. One Unit of activity is that amount which catalyses the dephosphorylation of 1 μmole of glycogen phosphorylase in one minute. The final concentration of microcystin-LR in the extracts — when added — was 280 nM.

phosphatase assay was greater than that of PP2A in the nuclear splicing extracts. As a further control, measurements were made of phosphatase levels in microcystin-LR-treated HeLa nuclear extracts under conditions where splicing was blocked. This confirmed that both PP1 and PP2A activities were fully inhibited. We note that the absolute levels of protein phosphatase activity detected in the HeLa nuclear extracts is consistent with our empirical observations of the concentrations of phosphatase inhibitors required to block splicing activity.

In summary, we show that both PP1 and PP2A activities are present in HeLa nuclear extracts, consistent with forms of these enzymes being the phosphatases required for splicing activity. However, it should be noted that these assays are performed using a standard substrate, and that the native forms of the phosphatases connected with splicing may have enhanced activity towards splicing factors and/or suppressed activity towards the standard substrate, as has been found for certain cytosolic forms of PP1 and PP2A (3, 29). Therefore, the data may not accurately reflect the relative levels of the specific PP1 and PP2A enzymes required for splicing.

## DISCUSSION

In this study we have presented evidence that reversible protein phosphorylation may play an important role in controlling pre-mRNA splicing. The highly specific phosphatase inhibitors tautomycin, microcystin-LR and okadaic acid were used to show that Ser/Thr-specific protein phosphatases are required for pre-mRNA splicing *in vitro*. The crude HeLa nuclear extracts used for *in vitro* splicing assays clearly contain one or more protein kinases which can utilise the magnesium and ATP present in the splicing reaction to inhibit both steps of pre-mRNA splicing. However, the extracts must also contain sufficiently high levels of endogenous protein phosphatases to compete with the kinase(s) and promote partial or complete dephosphorylation of proteins whose phosphorylation blocks splicing. When phosphatase inhibitors are added to the extract the balance between phosphorylation and dephosphorylation activities is changed and specific proteins therefore become phosphorylated to a degree which inhibits splicing. Interestingly, the phosphatase inhibitors uncouple spliceosome assembly from catalysis; i.e. tautomycin,

microcystin-LR and okadaic acid each block both of the catalytic steps of splicing but do not prevent the stable assembly of splicing complexes containing each of the U1, U2, U4/U6 and U5 snRNPs. Splicing activity could be restored to inhibited extracts by addition of exogenous, highly purified, mammalian Ser/Thr-specific protein phosphatases, confirming that protein dephosphorylation is essential for splicing *in vitro*. The data point to distinct protein phosphatases playing a major role during the first and second steps of splicing, indicating that at least two separate dephosphorylation events are required.

Previous detailed studies have established that tautomycin, microcystin-LR and okadaic acid are all highly specific inhibitors of the PP1 and PP2A type protein phosphatases (5–9). We therefore infer that it is forms of PP1 and PP2A which are required for splicing, although we cannot formally exclude the involvement of a novel, as yet undetected, phosphatase activity which is also sensitive to the same inhibitors. Consistent with a role for PP1 and PP2A in splicing, we show here that both activities are present in HeLa nuclear extracts and both are fully inhibited under conditions where splicing is blocked (Table 1). The data support a model in which a form of PP1 plays a major role for the first step of splicing and a form of PP2A plays a major role for the second step. This is based on the known properties of okadaic acid, which inhibits PP1 much less efficiently than PP2A (27), and our observation that okadaic acid preferentially blocks the second step of splicing unless used at high concentrations. The inhibitory effects of tautomycin and microcystin-LR also support this model since the former is more efficient at inhibiting PP1 than PP2A and the latter inhibits both PP1 and PP2A with similar efficiency (6, 7). When used to block splicing, tautomycin and microcystin-LR always inhibit both steps simultaneously. Although the purified catalytic subunits of either PP1 or PP2A alone were able to restore both steps of splicing to inhibitor-blocked extracts (Figure 4), this does not contradict the above model. Although PP1 appears to be the major phosphatase required for the first step of splicing and PP2A the major phosphatase required for the second step, a lesser contribution of PP2A at step one and PP1 at step two is not excluded. In this event it would not be surprising that high concentrations of either PP1 or PP2A could rescue both steps of splicing. Furthermore, it is also important to note that the catalytic subunits of phosphatases, in the absence of the additional regulatory subunits present in the native forms of enzymes, frequently show less restricted substrate specificities. This highlights the importance of purifying the native forms of protein phosphatases participating in splicing for future studies.

The present demonstration that Ser/Thr-specific protein dephosphorylation is required for both catalytic steps of splicing is consistent with the results presented in several recent studies (30, 31). Tazi *et al.* (30) reported that adenosine phosphorothioates (ATPαS and ATPγS) differentially inhibit the two catalytic steps of mammalian pre-mRNA splicing. The authors proposed that some splicing factors could be mammalian equivalents of the yeast RNA helicase-like proteins, while others might undergo phosphorylation–dephosphorylation cycles during spliceosome assembly and splicing. These authors also reported that okadaic acid specifically inhibited the second step of splicing. Here it is shown that okadaic acid, at high concentrations, inhibits both steps of splicing, rather than just the second step. However, a simple explanation for this difference is that Tazi *et al.* used okadaic acid at a concentration below that required to block the first step of splicing. Turcq *et al.* (31), cloned a gene required for RNA processing and splicing of *Neurospora* mitochondrial RNAs. The amino acid sequence deduced for this gene product shows similarity to yeast proteins that are important for cell-cycle progression and which are putative protein phosphatases or regulators of protein phosphatase activity. This evidence, although indirect, suggests that a role for protein phosphatases in the splicing mechanism may turn out to be an evolutionarily conserved feature. This would not be surprising as both the splicing machinery and protein phosphatases show an extremely high degree of evolutionary conservation.

This study shows that one or more protein factors, whose activity is required for completion of the first and second catalytic steps of splicing, are subject to regulation by the reversible phosphorylation of serine or threonine residues.

While the data indicate that distinct amino-acid targets must be dephosphorylated to allow the first and second catalytic steps of splicing, they do not distinguish whether these separate targets are located within the same or in different proteins. A major goal for future studies will now be to identify the phosphatase substrates which are required for the splicing mechanism. Such proteins could either be intrinsic components of snRNPs or splicing complexes or else could be extrinsic components which act to modulate the activity of splicing factors. The only major snRNP protein which is known to be phosphorylated *in vivo* is the U1 snRNP-specific 70 kD protein, which contains phosphoserine residues (32, 33). In this regard it is interesting that Tazi *et al.* have recently observed that thiophosphorylation of the U1 70 kD protein blocks the first step of splicing (personal communication).

The demonstration that Ser/Thr-specific protein phosphatase activity is required for pre-mRNA splicing *in vitro* raises interesting questions about the physiological role of reversible protein phosphorylation *in vivo*. It may be significant that protein phosphatase activity can uncouple spliceosome assembly from the catalytic steps of splicing as well as the two catalytic steps from each other. This could therefore allow spliceosome assembly and one or both steps of splicing to take place in different sub-nuclear locations. Alternatively, phosphorylation control could be used to modulate splicing activity *in vivo* in response to physiological stimuli, providing a sensitive mechanism for regulating production of spliced mRNA products. Yet another possibility is that the observed effects of phosphorylation are part of the cell-cycle control mechanism which acts to inhibit pre-mRNA splicing during mitosis. To distinguish between these and other possibilities it will now be important to identify and purify the different phosphatases and related kinases which act to regulate the pre-mRNA splicing machinery.

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