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Protein phosphatase 1 can modulate alternative 5′ splice site selection in a HeLa splicing extract

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Abstract Recent studies using HeLa in vitro splicing extracts have shown that changes in the relative concentrations of constitutive protein splicing factors can affect the choice between competing 5′ splice sites in alternatively spliced mammalian pre-mRNAs. Here we report that treatment of a HeLa splicing extract with human protein phosphatase 1 strongly inhibits formation of mRNA spliced to the distal 5′ splice site while stimulating relative use of the proximal 5′ splice site. This effect is not observed if spliceosomes assemble prior to protein phosphatase 1 treatment. These data show that alternative splicing in HeLa extracts can be mediated by changes in protein modification as well as by changes in the relative concentration of splicing factors. Changes in protein phosphorylation may thus provide a rapid mechanism for cells to respond to stimuli that require an alteration in alternative splicing patterns.

Key words: Protein phosphorylation; Protein phosphatase; RNA splicing; Alternative splicing

1. Introduction

The removal of introns from mRNA precursor transcripts (pre-mRNA) is an essential step during the expression of most genes in higher eukaryotes [1,2]. Intron removal takes place in the nucleus and is performed by a dedicated splicing machinery, termed the spliceosome, whose principal subunits are the U1, U2 and [U4/U6.U5] small nuclear ribonucleoprotein particles (snRNPs). Each snRNP comprises one or more small nuclear RNA and a set of proteins [3]. The snRNPs, together with additional non-snRNP protein splicing factors [4], assemble on pre-mRNAs in a stepwise pathway to form active spliceosomes. Detailed studies on the mechanism of splicing have been facilitated by the development of in vitro systems from both HeLa cells and budding yeast that accurately remove introns from exogenously added pre-mRNAs [2,5,6].

In addition to being an essential step for producing functional mRNA, splicing can be exploited as a versatile post-transcriptional regulatory mechanism. For example, by regulating the choice of alternative splice sites in a pre-mRNA, distinct mRNAs, and hence distinct protein products, can be generated from the same primary transcript. There are now many examples where such regulated splicing events are known to control biological processes [7,8]. Previous in vitro studies have shown that several members of the SR family of non-snRNP protein splicing factors can influence 5′ splice site choice in mammalian pre-mRNAs [9–16]. Specifically, in pre-mRNAs with alternative, competing 5′ splice sites, high concentrations of SR proteins favour the selection of proximal over distal 5′ splice sites. Conversely, high concentrations of the heterogeneous nuclear ribonucleoprotein (hnRNP) A1, favour use of distal over proximal 5′ splice sites [12]. Alternative splicing, at least in some cases, may thus be controlled by the balance between the relative concentrations of factors such as SR proteins and hnRNPA1 in the nucleus.

The SR proteins represent a family of nuclear phosphoproteins that share a common motif of repeating arginine-serine dipeptides and are highly conserved between mammals and drosophila [4,17,18]. They can be conveniently copurified by a two step salt precipitation method [19]. Interestingly, as well as their role in alternative splicing, SR proteins also have an essential function(s) in constitutive splicing. This has been demonstrated by the observation that HeLa S100 extracts, which are inactive for splicing, can be activated to splice pre-mRNA by adding exogenous SR proteins. There is also some functional redundancy between members of the SR protein family since splicing in HeLa S100 extracts can be activated by adding different SR proteins individually. There is good evidence that SR proteins are required at a very early stage of spliceosome assembly [11–16]. However, the mechanism by which they influence alternative splicing, or splicesome assembly, is still not understood.

Recently, evidence has emerged from studies using HeLa in vitro splicing extracts that reversible Ser/Thr-specific protein phosphorylation can influence both spliceosome assembly and the catalytic steps of the splicing reaction [20–22]. For example, addition of the specific protein phosphatase inhibitors okadaic acid, tautomycin or microcystin-LR to HeLa splicing extracts inhibits splicing but not spliceosome assembly [20,21]. A similar effect is obtained when a thiophosphorylated derivative of the U1 snRNP-specific 70 kDa protein is added to the extract [22]. In contrast, treatment of the HeLa extract with purified protein phosphatase 1 (PP1) potently inhibits spliceosome assembly (Mermound et al., EMBO J., in press). Collectively, these data indicate that protein phosphorylation may have a role in regulating constitutive pre-mRNA splicing events, both positively and negatively. In this study we report evidence that reversible protein phosphorylation may also have a role in regulating alternative splicing events. We demonstrate that for three separate pre-mRNAs which have alternative, competing 5′ splice sites, treatment of HeLa splicing extracts with purified PP1 in

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each case enhances relative utilisation of the proximal over the distal 5′ splice site.

2. Experimental

2.1. Splicing assays

HeLa nuclear splicing extracts were prepared as described by Dignam et al. [23] using the modifications described by Barabino et al. [24]. Pre-mRNA was prepared by in vitro transcription and used in splicing assays as described by Lamond et al. [25]. All splicing assays were carried out for 120 min at 30°C in a total reaction volume of 20 μl. RNA products of the splicing reaction were analysed by electrophoresis on a 7% denaturing polyacrylamide/8 M urea gel. Splicing complexes were analysed on non-denaturing agarose-acrylamide composite gels, as described in Lamond et al. [26].

2.2. Analysis of alternatively spliced RNAs

RNAs spliced to the cryptic 5′ splice site were eluted from the gel in 300 μl of 0.2 M sodium acetate, pH 7, 0.1 mM EDTA in the presence of 5 μg tRNA carrier. After elution overnight at room temperature, the RNA was extracted once with an equal volume of phenol/chloroform (1:1, v/v), and precipitated from the supernatant by addition of 2.5 volumes ethanol. The eluted RNAs were copied into cDNA using AMV Reverse Transcriptase as described in Sambrook et al. [27], amplified by the PCR method [28] and sequenced directly without subcloning of the amplified product [29].

2.3. Isolation of SR proteins

SR proteins were isolated from HeLa cells according to the method of Zahler et al. [19] and analysed by Coomassie staining after PAGE separation. As a functional control, isolated SR proteins were shown to stimulate splicing in a HeLa S100 extract (data not shown). The SR proteins were added to HeLa nuclear extracts prior to addition of pre-mRNA at the start of the splicing assays.

2.4. Preparation of PP1

Cloned human PP1γ [30] was expressed in *E. coli* and purified to homogeneity as described by Alessi et al. [31]. One unit of PP1 activity is defined as the amount of activity that catalyses the dephosphorylation of 1nmole of glycogen phosphorylase in 1 minute. PP1γ was stored at a concentration of 30–60 units/μl in 50% glycerol, 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.1% mercaptoethanol. Prior to use the enzyme was diluted into 20 mM HEPES pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA. PP1γ was added to the HeLa extract at a final concentration of 0.1 units/μl prior to addition of the pre-mRNA at the start of the assay.

2.5. Construction of plasmids encoding pre-mRNAs with alternative 5′ splice sites

Alternatively spliced pre-mRNAs BC1 and BC2 were prepared by in vitro transcription of *Pvu*II cut plasmids pBSBC1 and pBSBC2 respectively, using phage T3 RNA polymerase. The pBSBC1 and pBSBC2 constructs were made by modifying the plasmid pBSAL4 [25]. Briefly, pBSAL4 was cut at the unique *Sty*I site in the intron sequence and double stranded DNA oligonucleotides BC1 (5′-CATGTAGCTTCGAAACAGGTAAGTCTCGAC-3′) or BC2 (5′-CATGTAGCTTGCTCTAAGGTAAATATCGAC-3′) inserted. Recombinants with correctly oriented inserts were isolated and their structures confirmed by DNA sequencing using standard methods.

3. Results

We have observed that addition of exogenous SR proteins to HeLa nuclear extract can activate a previously silent cryptic 5′ splice site within an intron derived from the major late transcript of adenovirus (adeno pre-mRNA) (Fig. 1A). This cryptic 5′ splice site was mapped and the alternatively spliced mRNA product sequenced (data not shown). The cryptic site (i.e. 5′ splice site proximal to the 3′ exon) has a 5/8 match to the consensus mammalian 5′ splice site sequence [1,2], while the constitutively used 5′ splice site (i.e. 5′ splice site distal to the

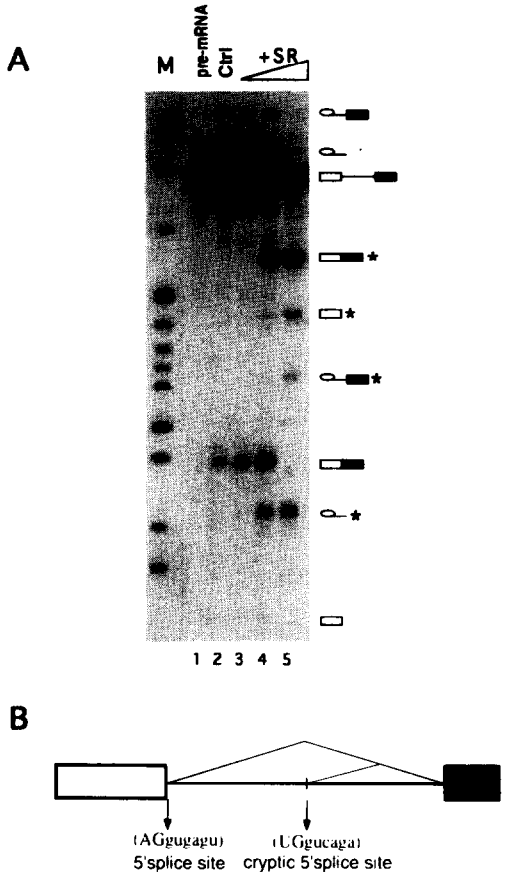

Fig. 1. SR proteins activate a cryptic 5′ splice site in adeno pre-mRNA. (A) A pre-mRNA substrate derived from the major late transcription unit of adenovirus [22] was incubated in HeLa nuclear splicing extract either without exogenous SR proteins (lane 2) or in the presence of 1.5 μg, 3 μg or 5 μg exogenously added SR proteins (lanes 3–5). The identity of the RNA intermediates and products of the splicing reaction are cartooned at the right hand side of the figure with all species derived from use of the proximal (i.e. cryptic) 5′ splice site marked by an asterisk. Lane 1 shows unspliced adeno pre-mRNA and the lane marked M shows DNA size markers derived from an *Msp*I digest of pBR322 DNA end-labeled using Klenow polymerase and [α-^{32}P]dCTP. (B) The alternative 5′ splice sites in adeno pre-mRNA are illustrated along with the respective sequences at the competing proximal and distal 5′ splice junctions.

3′ exon) has a 7/8 match to the consensus (Fig. 1B). The effect of the SR proteins is complex, since addition of lower levels of SR proteins stimulates use of the distal 5′ splice site, while at higher levels they activate the proximal (i.e. cryptic) 5′ splice site and reduce utilisation of the distal site (Fig. 1A, lanes 2–5 and other data not shown). At the highest concentration used, addition of the exogenous SR proteins results in inactivation of the distal 5′ splice site while the proximal 5′ splice site remains active (Fig. 1A, lane 5). These results are consistent with previous reports showing the effect of SR proteins on alternative splicing, although in all cases the mechanism involved is unknown.

We have recently observed that constitutive splicing and spliceosome assembly is inhibited when HeLa nuclear extracts are treated with human Ser/Thr-specific protein phosphatase 1γ (PP1γ) (Mermoud et al., EMBO J., in press). Remarkably, when this experiment is repeated in an extract where addition

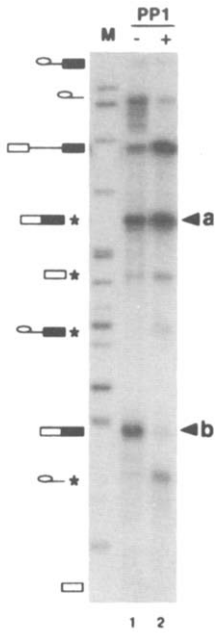

Fig. 2. PP1γ differentially inhibits competing 5′ splice sites. The adeno pre-mRNA was incubated in HeLa nuclear splicing extract supplemented with 3μg of exogenous SR proteins either in the absence (lane 1), or presence (lane 2), of 0.1 units/μl (final concentration) of purified, *E. coli* expressed human PP1γ. The identity of the RNA intermediates and products of the splicing reaction are cartooned at the left hand side of the figure with all species derived from use of the proximal 5′ splice site marked by an asterisk. The mRNA products formed using the proximal (a) and distal (b) 5′ splice sites are marked by arrowheads at the right hand side of the figure. The lane marked M shows DNA size markers derived from an *Msp*I digest of pBR322 DNA end-labeled using Klenow polymerase and [α-^{32}P]dCTP.

of SR proteins has activated cryptic splice site utilisation in adeno pre-mRNA, PP1γ inhibits splicing from the distal, but not from the proximal, 5′ splice site (Fig. 2, compare levels of mRNA spliced to the proximal 5′ splice site (arrowhead a) and to the distal 5′ splice site (arrowhead b) in lanes 1 and 2). This indicates that utilisation of the two alternative 5′ splice sites in the adeno pre-mRNA is differentially sensitive to protein dephosphorylation. Since the PP1γ used is a human enzyme that was expressed in *E. coli* from the cloned PP1γ gene and purified to homogeneity [30,31], the differential inhibition of proximal and distal 5′ splice sites cannot be due to exogenous mammalian splicing factors contaminating the PP1γ preparation. Control experiments confirm that it is also not caused by *E. coli* proteins (data not shown). We conclude, therefore, that PP1 treatment is responsible for changing the relative utilisation of the competing 5′ splice sites.

We next addressed whether PP1γ had a similar effect on other pre-mRNAs that utilise alternative 5′ splice sites. Therefore, two constructs were made in which either the consensus 5′ splice site sequence (BC1), or the SV40 t antigen 5′ splice site sequence (BC2), was inserted into an intron derived from the rabbit β-globin gene (Fig. 3A). In each case, the pre-mRNA has a distal 5′ splice site corresponding to the normal β-globin sequence and a proximal 5′ splice site corresponding to the inserted sequence. As in the previous experiments, use of the proximal 5′ splice site in both constructs is increased by addition of SR proteins to the splicing assay (Fig. 3B). The identities of the alternatively spliced products were confirmed in each case by sequence analysis (data not shown). Adding low levels of exogenous SR proteins again increases use of both proximal and distal splice sites, while addition of higher levels of SR proteins increases the fraction of pre-mRNA spliced to the proximal 5′ splice site (Fig. 3B). Treatment of the HeLa splicing extract with PP1γ resulted in preferential inhibition of the distal

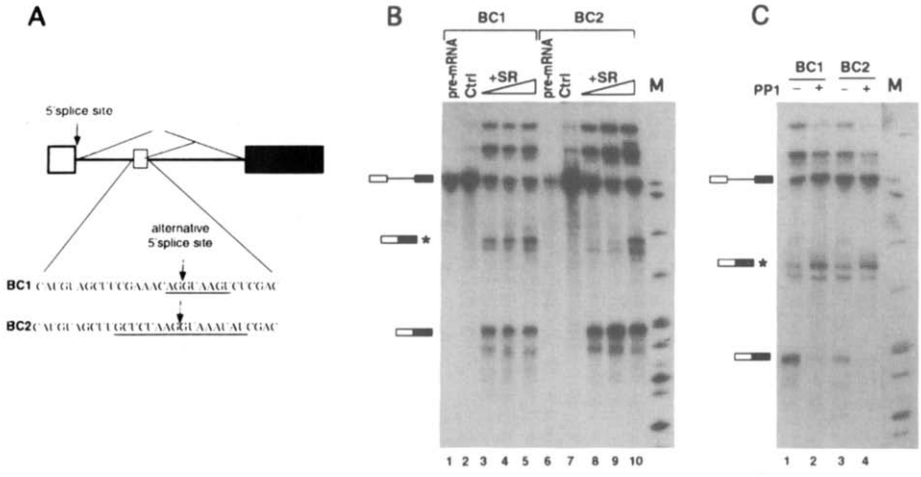

Fig. 3. PP1γ selectively inhibits use of distal 5′ splice sites in separate alternatively spliced pre-mRNAs. (A) Cartoon illustrates the structures of the alternatively spliced pre-mRNA constructs BC1 and BC2. In both constructs the distal 5′ splice site corresponds to the natural 5′ splice site of the large intron of rabbit β-globin, while the proximal 5′ splice sites correspond to either the 5′ splice site consensus sequence (BC1), or the SV40 t antigen 5′ splice site. (B) Splicing assays were carried out using both BC1 (lanes 1–5) or BC2 (lanes 6–10) pre-mRNAs, either in the absence of exogenous SR proteins (lanes 2 and 7), or in the presence of 1.5 μg (lanes 3 and 8), 3 μg (lanes 4 and 9) or 5 μg (lanes 5 and 10) exogenous SR proteins. Lanes 1 and 6 show markers corresponding to the unspliced BC1 and BC2 pre-mRNAs, respectively. The lariat intermediates and products migrate more slowly than the unspliced pre-mRNA as previously described for the parent globin construct [25]. (C) Splicing assays using either BC1 (lanes 1 and 2), or BC2 (lanes 3 and 4), pre-mRNAs were carried out in HeLa extracts supplemented with 3 μg SR proteins either in the absence (lanes 1 and 3), or presence (lanes 2 and 4), of 0.1 units/μl (final concentration) of purified, *E. coli* expressed human PP1γ. The cartoons at the left hand side of gels B and C show unspliced pre-mRNA and mRNA formed using either the proximal 5′ splice site (asterisk) or distal 5′ splice site. Lanes M correspond to DNA size markers derived from an *Msp*I digest of pBR322 DNA end-labeled using Klenow polymerase and [α-^{32}P]dCTP.

5′ splice site for both constructs (Fig. 3C). Although the effect is less dramatic than seen with adeno pre-mRNA, the ratio of mRNA product spliced to the proximal, rather than distal, 5′ splice site of both constructs is increased in extracts incubated with PP1γ. PP1γ thus has a qualitatively similar effect on alternative splice site selection for three separate proximal 5′ splice site sequences and in the context of two distinct intron backgrounds.

Splicing of pre-mRNA is a multi-step process involving two, distinct catalytic steps preceded by a complex pathway of spliceosome assembly [1,2,5,6]. The requirement for assembly of spliceosomes on pre-mRNA produces a lag phase of 15–20 min before spliced products are detected during in vitro splicing assays. Therefore, we next compared the effect of treating the HeLa splicing extract with PP1γ either prior to spliceosome assembly (i.e. adding PP1γ and pre-mRNA simultaneously to the extract), or after assembly, but prior to catalysis (i.e. adding PP1γ 15 min after adding pre-mRNA to the extract). The time of addition of PP1γ significantly affected its ability to influence 5′ splice site selection (Fig. 4A). Thus, the same amount of enzyme that caused preferential utilisation of the proximal 5′ splice site when added at the start of the reaction, (Fig. 4A, lane 6) had little or no effect on 5′ splice site choice when added after 15 min (Fig. 4A, compare lane 9 with lanes 3 and 6). Parallel native gel analysis confirmed that when PP1γ was added to the splicing reaction after 15 min spliceosomes had already assembled (Fig. 4B, lane 8). Interestingly, treatment of the HeLa extract with PP1 also resulted in much of the splicing complexes accumulating at the top of the native gel, suggesting that PP1 treatment may lead to some form of higher order complexes being produced (Fig. 4B, lanes 5,6,8 and 9).

These data demonstrate that the PP1γ does not simply have a non-specific inhibitory effect on the catalysis of splicing at certain splice sites. The data favour a model in which PP1γ either prevents spliceosome assembly at distal 5′ splice sites, or causes the complexes assembled at distal 5′ splice sites to remain inactive. The resistance to PP1γ treatment after spliceosome assembly has taken place could result either from the target proteins whose activity is affected only being required at early stages of spliceosome assembly, or, alternatively, from spliceosome assembly preventing access of PP1γ to the key targets.

4. Discussion

We have shown using a HeLa in vitro splicing extract that a protein phosphorylation mechanism can influence the relative utilisation of competing 5′ splice sites in alternatively spliced mammalian pre-mRNAs. Treatment of the HeLa extract with PP1γ increases the proportion of RNA that is spliced to the 5′ splice site proximal to the 3′ exon. The mechanism whereby PP1γ differentially inhibits use of distal 5′ splice sites remains to be determined. The mechanism of action of SR proteins, which also result in a preferential use of proximal 5′ splice sites, is similarly unknown [4,9–19]. Interestingly, SR proteins are highly phosphorylated in vivo and are required for early steps during spliceosome assembly [9–19]. The alternative splicing activity of SR proteins could thus be influenced by PP1γ. The level of SR proteins used in these experiments is likely higher than the concentration in vivo. This may reflect differences in the efficiency of the splicing reaction in the in vitro extract compared with the splicing reaction that takes place on nascent

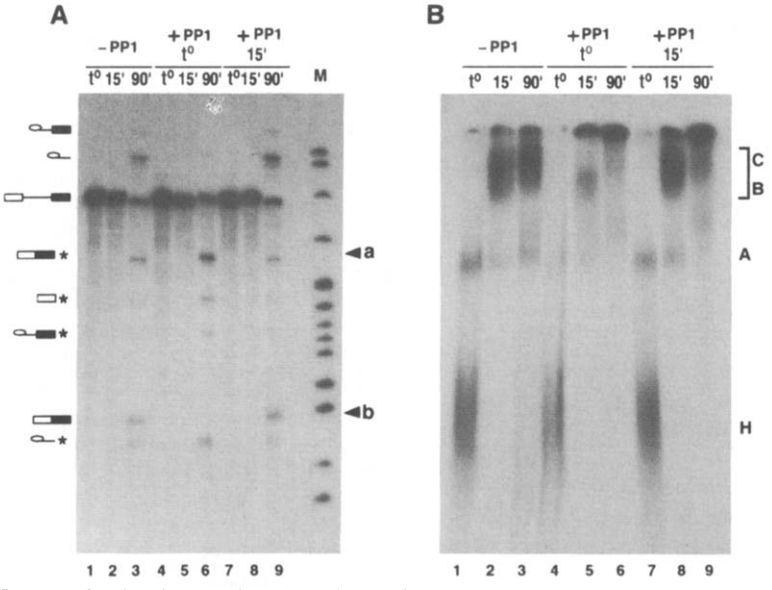

Fig. 4. Time of PP1γ addition influences 5′ splice site selection. (A and B) Splicing assays, using adeno pre-mRNA, were carried out in HeLa nuclear splicing extract supplemented with 3 μg exogenous SR proteins, either in the absence (lanes 1–3), or in the presence (lanes 4–9), of 0.1 units/μl (final concentration) of human PP1γ. Analysis of RNA products is shown in panel A and analysis of splicing complexes formed in the same reactions in panel B. In the reactions shown in lanes 4–6, PP1γ was added to the splicing reaction prior to the pre-mRNA at the start of the assay, while in the reactions shown in lanes 7–9, the enzyme was added 15 min after the start of the assay. RNAs from the separate reactions were analysed at t^0 (lanes 1,4 and 7), after 15 min incubation at 30°C (lanes 2,5 and 8) and after 90 min incubation at 30°C (lanes 3,6 and 9). The identity of the RNA intermediates and products of the splicing reaction in panel A are cartooned at the left hand side of the figure with all species derived from use of the proximal 5′ splice site marked by an asterisk. The mRNA products formed using the proximal (a) and distal (b) 5′ splice sites are marked by arrowheads at the right hand side of the figure. The lane marked M shows DNA size markers derived from an *Msp*I digest of pBR322 DNA end-labeled using Klenow polymerase and [α-^{32}P]dCTP. (B) In the native gel B and C indicate spliceosome complexes, A indicates pre-spliceosomes and H unspecific complexes.

transcripts in vivo. However, if their activity is indeed regulated through their phosphorylation state, the concentration of active SR proteins in the splicing extract may in fact be within the physiological range.

Recent studies have shown that the heterogeneous nuclear ribonucleoprotein (hnRNP)A1 acts antagonistically to SR proteins, i.e. increasing concentration of hnRNP A1 promotes selection of distal over proximal 5′ splice sites [12]. Since hnRNP A1 is also a phosphoprotein in vivo [32], downregulation of hnRNP A1 by PP1γ treatment may also contribute to the observed effects on alternative splicing. Another phosphoprotein that plays an important role during early steps of spliceosome assembly is the 70 kDa antigen which is a specific component of U1 small nuclear ribonucleoprotein (U1 snRNP) [3]. Thiophosphorylation of U1 70 kDa has been shown to inhibit splicing catalysis, but not spliceosome assembly, for a constitutively spliced mammalian pre-mRNA [22]. Although U1 snRNP has so far not been implicated as an alternative splicing factor, it is known to bind to 5′ splice sites and is essential for splicing [1,2,5,6]. It is possible, therefore, that the phosphorylation state of the 70 kDa protein could contribute to the differential selection of proximal and distal 5′ splice sites. Whether SR proteins, hnRNP A1, U1 70 kDa or other splicing factors are responsible for the effect of PP1γ on alternative splicing remains to be addressed in future studies.

The observation that treatment of HeLa splicing extracts with human PP1γ selectively inhibits formation of mRNA spliced to a distal 5′ splice site could result either from PP1γ acting directly on one or more splicing factors, or from an indirect effect of PP1γ (e.g. modulation of a kinase in the HeLa extract), which in turn affects the activity of splicing factors. Since the HeLa splicing system corresponds to a crude nuclear extract that contains both endogenous kinase and phosphatase activities, it is possible that a phosphorylation cascade is taking place and that PP1 treatment affects this cascade rather than acting itself on a splicing factor. It is also possible that the effects we observe in vitro using purified PP1 could be mediated in vivo by a different protein phosphatase with overlapping substrate specificity. However, despite the crude nature of the HeLa extract, the inhibitory effects of PP1 and of phosphatase inhibitors on splicing are clearly very specific.

It is known that changes in gene expression in vivo can be induced by signal transduction pathways that influence phosphorylation activity in the nucleus. The data presented here raise the possibility that this may be achieved, in certain cases, via changes in alternative splicing patterns. A change in the activity of one or more splicing factors promoted by a protein phosphorylation mechanism, rather than a change in the concentration of splicing factors within cells, could provide a rapid and flexible way of regulating gene expression at the post-transcriptional level. Future studies will, therefore, be directed towards analysing the physiological significance of protein phosphorylation for alternative splicing in vivo.

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