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Original Article

Safeguarding of pre-mRNA splicing by Prp4 kinase is based on formation of transient structures

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ABSTRACT

In fission yeast, Prp4 kinase activity is required for recognition of introns with weak splice sites. In this context, we analyzed the consequences of mutations within the branch sequence. We found that only changes in the nucleotide transiently interacting with a pseudouridine in snRNA U2 and one double mutation in the branch sequence lead to the requirement of Prp4 kinase activity. The results presented here and previously let us propose that Prp4 kinase activity is part of a safeguarding process that facilitates formation of transient RNA duplexes between pre-mRNAs and snRNAs to form the pre-spliceosome.

KEYWORDS: Prp4 kinase, intron recognition, RNA-RNA interactions, pseudouridine, SR proteins.

INTRODUCTION

Introns are removed from pre-mRNAs by the spliceosome, a highly dynamic macromolecular complex consisting of five ribonucleoprotein particles [snRNPs; U1, U2, U5, U4/U6], and additional non-snRNP proteins [1]. How are introns recognized by the spliceosome? A large body of evidence indicates that recognition of introns involves transient base-pairing between snRNA U1 and the 5´ splice site (5´ SS), as well as between snRNA U2 and the branch sequence (BS), to ensure proper recognition (Fig. 1A) [2-6]. In mammalian cells introns can be recognized according to the exon definition model as well as to the intron definition model, whereas in fungi the introns are recognized according to the intron definition model *via* interaction of the spliceosomal subparticles across the intron [7]. It is also understood that pre-mRNA splicing is part of ongoing gene expression. Therefore introns are mostly spliced during transcription leading to the recognition of splice sites in space and time [8].

As a first step it is thought that snRNP U1 binds with the 5´ end of its RNA, the 5´ SS, through complementary base pairing interactions [3, 9, 10]. Then, spBpb1 (hsSF1) binds to the BS and a heterodimer consisting of spPrp2 (hsU2AF2) and spUaf2 (hsU2AF1) labels the 3´ AG by binding to the often pyrimidine-rich region upstream of it thereby connecting to the BS [11-13]. This state is known as the E-complex and plays an important role during intron recognition since it marks the 5´ and 3´ SS of the intron. Subsequently, the interaction of spBpb1 (hsSF1) with the BS is disrupted and spBpb1 is replaced by snRNP U2 undergoing transient base-pairing between snRNA U2 and the BS including the bulged out branchpoint adenosine to form a prespliceosome [2, 4]. Then the NineTeen Complex (NTC) and a tri-snRNP consisting of U5 and U4/U6 base-paired join and the pre-catalytic spliceosome is formed. This state is known as the B-complex. After rearrangements, snRNPs U1 and U4 and the proteins spPrp2 and spUaf2 are released, the spliceosome is activated and the transesterification reactions occur [14-16].

In *Schizosaccharomyces pombe*, the last three exonic and the first six intronic nucleotides of the pre-mRNA can be involved in forming transient complementary base pairing interactions with the

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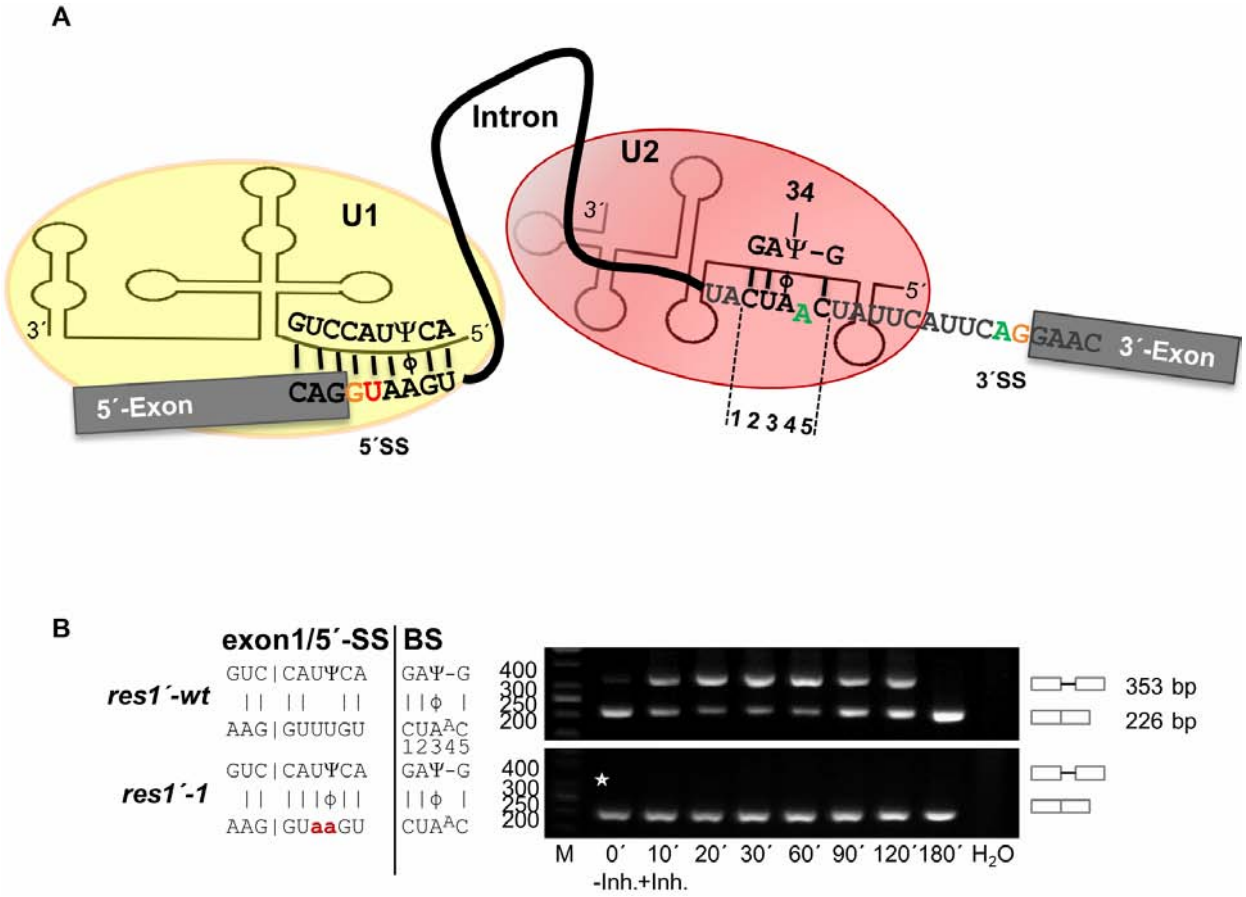

Fig. 1. Intron recognition by snRNP particles U1 and U2. (A) Proposed base-pairing between the *res1'-1* exon1/5' SS region and intron branch sequence CUAAC with snRNA U1 and snRNA U2, respectively. Ψ indicates the pseudouridine 3 nucleotides from the 5' end of snRNA U1 and the pseudouridine 34 nucleotides from the 5' end of snRNA U2, which is thought to base-pair with the A at position 3 of the branch sequence. Numbering of the branch sequence in the pre-mRNA is indicated. **(B)** RT-PCR analysis was performed in the absence (-Inh.) and presence (+Inh.) of inhibitor at the indicated times. H_2O: negative control without template. The scheme on the left side of the images shows details of the interactions between exon1/5' SS and snRNA U1 and the branch sequence and snRNA U2. Small letters in red indicate mutations. |, Watson-Crick base-pairing; Ψ, pseudouridine; ϕ, wobble base-pairing (Ψ-A, Ψ-G, Ψ-U, Ψ-C); Asterisks indicate expected positions of fragments of pre-mRNA. The numbers on the left side of the images show the sizes of DNA fragments (bp). M, DNA size marker.

first nine nucleotides of the snRNA U1, whereas the third nucleotide of the snRNA U1 is a pseudouridine (Ψ) (3'-GUCCAUΨCA-5') [17-19]. The sequence GUAAGU appears to be the most frequent intronic 5´ SS (27.7%) [20, 21]. The exonic sequences are degenerate as these are part of the coding sequence. The nine nucleotides which can potentially form complementary base pairing interactions with snRNA U1 can deviate from the optimal sequence CAG/GUAAGU. These introns, displaying weak 5' SSs, require the activity of Prp4 kinase to be spliced out efficiently; accordingly, these were termed Prp4-dependent introns. Introns with strong 5´ SSs are spliced efficiently in the absence of Prp4 kinase activity, and are thus termed Prp4-independent introns [19].

Currently available bioinformatic data indicate that BSs are degenerate in fungal and mammalian cells [7, 22]. *S. pombe*, for example, has 13 different BSs among 4603 introns, with CUAAC (1943) and CUAAU (1038) the most frequent [20]. These five nucleotides might interact transiently with snRNA U2, as shown in Fig. 1A, whereas the adenosine (A) at position 4 is thought to bulge out as the branchpoint

to facilitate the first transesterification reaction [23]. In addition, the snRNA U2 sequence between *Homo sapiens* and *S. pombe* is remarkably conserved, for example, showing an identical sequence between 30 to 40 nucleotides upstream of the 5´ end [24]. In *H. sapiens* the nucleotides 34, 37 and 39 seem to be pseudouridines (Ψ), whereas in *S. pombe* the nucleotides 34 and 37 appear to be pseudouridinylated [18, 25]. A topic of special interest in this context is the effect of the interaction between the Ψ at position 34 in snRNA U2, which interacts with the nucleotide at position 3 of the BS and is the nearest neighbor interaction to branchpoint A (Fig. 1A, [23]). Ψ in this position appears to be essential to arrange the branchpoint A for the first transesterification reaction [26, 27].

In mammalia it is known that, in constitutive splicing SR and SR-like proteins are needed during intron recognition at the 5´ exon/5´ SS to interact with hsU1-70K (spUsp101) as well as at the 3´ SS to interact with hsU2AF1 (spUaf2) [28]. These proteins are called serine/arginine-rich (SR) proteins and obviously facilitate transient stabilization between the pre-mRNA and snRNAs [29-32]. In *S. pombe* there are two SR proteins known, spSrp1 (hsSRSF2) and spSrp2 (hsSRSF4/5/6) [7, 33, 34]. spSrp2 is known to be phosphorylated by Prp4 kinase while spSrp1 is not [35]. One of the SR-like proteins in *S. pombe* is spRsd1 (hsRBM39/23). This protein is known to bridge the U1-U2 snRNP interaction during prespliceosome formation. Therefore it interacts with the U1 snRNP protein spUsp102 (hsU1A) and the helicase spPrp11 (hsDDX46) which itself binds to snRNP U2 [36].

In this report, we show by extensive mutational analysis of the BS CUAAC that inhibition of Prp4 kinase leads to the timewise accumulation of pre-mRNA after mutating position 3 of the BS, which is opposite the Ψ at position 34 in snRNA U2. As a matter of fact, when the complementarity of the 4 nucleotides to snRNA U2 around the BP was circumvented or compromised, the BS is either not recognized or considered as a weak BS as shown by the timewise accumulation of pre-mRNA when Prp4 kinase is inhibited. In addition, we show here the *in vitro* phosphorylation of spUsp101 (hsU1-70K), spBpb1 (hsSF1), spSrp2 (hsSRSF4/5/6) and spRsd1 (hsRBM39/23) by Prp4 kinase which makes them good candidates to be involved in the safeguarding system leading to proper recognition of introns displaying weak SSs and BSs. This idea will be further explored and discussed in this report.

MATERIALS AND METHODS

Yeast strains and growth conditions

The standard genetic and molecular techniques used in this study were described previously [37, 38]. All strains used in this study are listed in Table 1.

Construction of an analogue-sensitive Prp4 kinase

The analogue-sensitive *prp4-as2* kinase allele was generated as described previously [19].

Construction of the reporter gene *res1´*

To construct the reporter gene, *res1´* was fused to the thiamine-repressible *nmt1-8* promoter by cloning the open reading frames into vector pML81HA [19, 39, 40]. To distinguish between the wt *res1* transcript and the *res1´* transcript at the *leu1* locus by reverse transcription-polymerase chain reaction (RT-PCR), a *Hind*III restriction site was introduced into exon 1 of the gene. The forward primer (res1_Mut_F) detects the *Hind*III site.

Reverse transcription-PCR

To determine the pre-mRNA splicing patterns of intron-containing genes, RNA was extracted from whole-cell extracts. For RT-PCR, RNA was treated with RQ1 RNase-free DNase (Promega) to eliminate possible DNA contaminants. Five micrograms of RNA were treated with 2.5 U RQ1 DNase in reaction buffer containing 40 mM Tris–HCl (pH 8.0), 10 mM $MgSO_4$, and 10 mM $CaCl_2$. After incubation for 10 min at 37 °C, RQ1 DNase was inactivated by the addition of 2 mM EGTA (pH 8.0) and heating to 80 °C for 10 min. RNA was reverse transcribed and cDNA was amplified using *Tth* reverse transcriptase (Roboklon, EURX). RNA was incubated for 3 min at the calculated annealing temperature of 56.2 °C in the presence of 1× *Tth* RT Buffer, 0.25 mM dNTP mix, 20 pmol reverse primer, 2 mM $MnCl_2$, and 0.25 U/ml Tth RT, followed by incubation at 70 °C for 25 min. PCR mix containing 1× PCR-Buffer Pol A, 80 pmol reverse primer (res1_E2-R: 5'-AAGGCCGGGAAAATG ACTTTTT-3'), 100 pmol forward primer (res1_ Mut-F: 5'-GAAAAAATTCAAGGTGGTTGTG GAAGC-3'), and 2 mM $MgCl_2$ was then added.

Table 1. *Schizosaccharomyces pombe* strains used in this study.

Strain	Genotype
971	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-1 ura4-D18*
991	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-WT ura4-D18*
998	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-12 ura4-D18*
999	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-2 ura4-D18*
1000	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-11 ura4-D18*
1001	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-3 ura4-D18*
1006	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-14 ura4-D18*
1097	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-13 ura4-D18*
1098	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-15 ura4-D18*
1101	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-4 ura4-D18*
1102	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-7 ura4-D18*
1103	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-6 ura4-D18*
1104	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-5 ura4-D18*
1108	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-10 ura4-D18*
1109	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-9 ura4-D18*
1110	*h^{-S} prp4 int::prp4-as2-kan^{R} leu1-32 int::pML81HA-res1´-8 ura4-D18*

cDNA was amplified with 45 cycles of 94 °C for 30 s, 56.2 °C for 30 s, and at 72 °C for 60 s. PCR products were resolved on 2% agarose gels.

Plasmids and protein production

For protein production the pGEX-6p-1 vector (Amersham) was used. The cDNA of the genes was amplified and cloned in the multiple cloning site (MCS) in frame with the glutathione S-transferase (GST) tag. Recombinant N-terminal GST-tagged proteins spPrp4 kinase, spUsp101, spUsp102, spUsp103, spUsp107, spPrp2, spBpb1, spUaf2, spSrp1, spSrp2 and spRsd1 were expressed in the *Escherichia coli* strain BL21, purified *via* the GST tag using Glutathione-Sepharose 4B (GE Healthcare) and eluted using PreScission protease (GE Healthcare). The amount was measured with Bradford reagent.

In vitro kinase assay

Purified Prp4 kinase was incubated at 30 °C for 30 min. with the potential purified substrates. For the kinase reaction the respective potential substrate was mixed with the kinase, in a ratio of 2.5: 1 in a total volume of 20 µL consisting of 20 mM HEPES-KOH (pH 7.5, 1 mM DTT), 50 µM ATP, 1 mM $MgCl_2$ and 4 µCi $\gamma[^{32}P]$ ATP. The reaction was stopped using 5x SDS sample buffer and after incubation for 5 min. at 100 °C. Then the samples were applied to a SDS gel and separated. Protein phosphorylation was detected by autoradiography.

RESULTS AND DISCUSSION

Several years ago, we observed that introns of *S. pombe* are recognized and removed even when introduced into a naturally intronless gene indicating that mostly intronic sequences might be responsible for intron recognition [41]. Recently, we constructed and examined the *res1´* reporter gene, which was integrated at the *leu1* locus and contained a 127 nt long intron. In this study, we confirmed the idea that mostly intronic sequences seem to be involved in intron recognition. We found that the Prp4 kinase dependency of this intron was not related to the location of the gene on the chromosome, but was instead dependent on the number and specific base-pairing interactions between snRNA U1 and the 5´ SS, as well as snRNA U2 and the BS [19]. Mutational analysis of the 5´ SS revealed that increasing the number of putative transient interactions between snRNA U1 and the 5´ SS converted the intron from Prp4-dependent to Prp4-independent (Fig. 1B, *res1´-wt* vs. *res1´-1*).

To determine the impact of mutations in the BS on Prp4-dependent splicing, we used as an initial construct the *res1´* reporter gene, which has a strong exon1/5´ SS region (AAG/GUAAGU) and a strong BS (CUAAC) that is spliced out efficiently in the presence or absence of 1NM-PP1, an inhibitor of the analogue sensitive Prp4 kinase (Fig. 1B, *res1´-1*). We mutated each position of the BS CUAAC in the intron of the *res1´* reporter gene, and monitored splicing efficiency of the mutant intron in the absence (-Inh) and presence (+Inh) of inhibitor *via* RT-PCR time dependently. When we introduced a G at position 1 or 5, which precludes Watson-Crick base-pairing with snRNA U2 at these positions, the intron was still spliced Prp4-independently, although splicing efficiency was slightly reduced in the presence of inhibitor (Fig. 2, *res1´-2 & res1´-3*). When we introduced an A or a U at these positions, the intron was spliced Prp4-independently at all time points (Fig. 2, *res1´-4-7*). Thus, these positions are not essential for intron recognition when mutated separately. However, when these two positions were mutated simultaneously, the intron behaved very differently. When positions 1 and 5 were replaced with Gs, the intron was no longer recognized, indicating that disruption of two typical base-pairing interactions between the BS and the two Gs of the snRNA U2 prevented intron recognition (Fig. 3A, *res1´-8*). This observation implies that base-pairing interactions of only two nucleotides, at positions 2 and 3 of the BS, with snRNA U2 are not sufficient for intron recognition. By contrast, mutation of these positions to As yielded a Prp4-dependent intron: without kinase inhibition, the intron was spliced efficiently, but after adding the inhibitor, pre-mRNA accumulated between 10 and 90 min (Fig. 3A, *res1´-9*). When we introduced Us at these positions, compromising

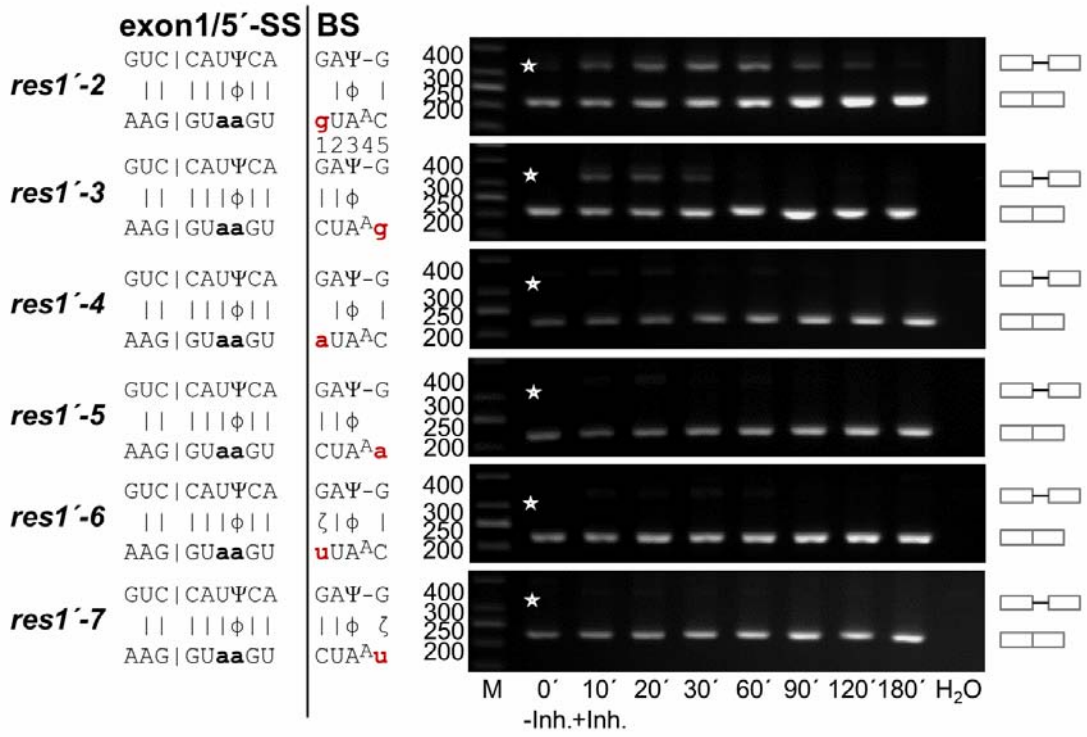

Fig. 2. Mutations at position 1 or 5 of the branch sequence do not change Prp4 independency. RT-PCR analysis was performed in the absence (-Inh.) and presence (+Inh.) of inhibitor at the indicated times. H_2O: negative control without template. The scheme on the left side of the images shows details of the interactions between exon1/5' SS and snRNA U1 and the branch sequence and snRNA U2. Small letters in red indicate mutations. |, Watson-Crick base-pairing; Ψ, Pseudouridine; ϕ, wobble base-pairing (Ψ-A, Ψ-G, Ψ-U, Ψ-C); ζ, wobble base-pairing (G-U). Asterisks indicate the expected positions of fragments of mRNA or pre-mRNA. The numbers on the left side of the images show the sizes of DNA fragments (bp). M, DNA size marker.

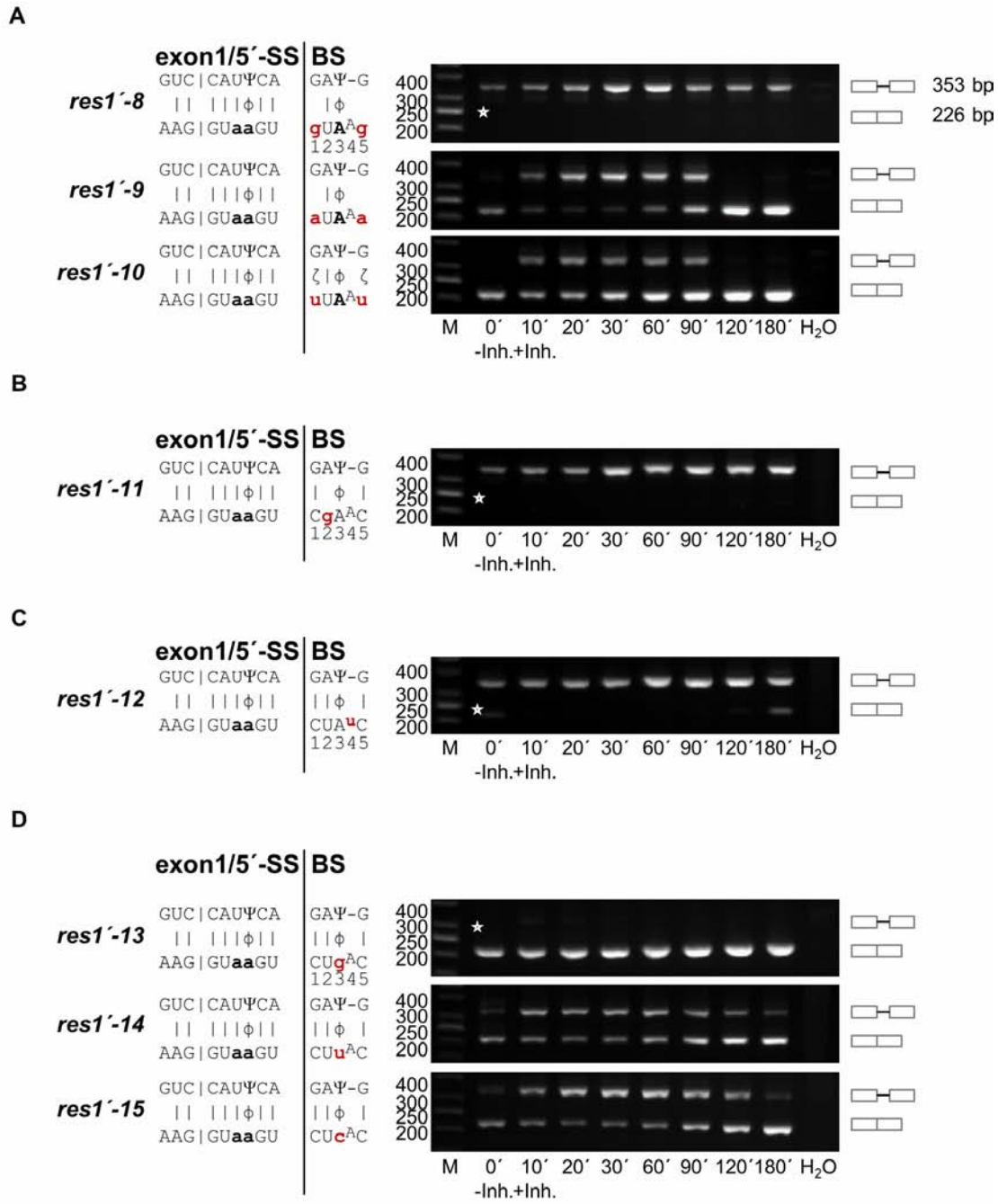

Fig. 3. Mutations of the branch sequence leading to Prp4 dependency. (A) Mutations in position 1 and 5 as indicated. **(B)** Mutation in position 2. **(C)** Mutation at the branchpoint (BP). **(D)** Mutations at position 3. The requirement for Prp4 kinase activity depends on the strength of the interaction between Ψ and the nucleotide at position 3 of the branch sequence. RT-PCR analysis and conditions as described in Fig. 2.

typical Watson-Crick base pairing, pre-mRNA appears to accumulate slightly after addition of the kinase inhibitor. However accumulation of pre-mRNA and disappearance of mRNA during the first 60 minutes of inhibition are less distinct and the intron was spliced Prp4-independently, but splicing efficiency is clearly decreased over this time period (Fig. 3A, *res1´-10*). These results

are consistent with the fact that G can form a wobble base-pair interaction with U that has a comparable thermodynamic stability to Watson-Crick base pairs [42], whereas the interaction with As in these positions led to a typical Prp4-dependent appearance of pre-mRNA followed by complete recovery of splicing (Fig. 3A, *res1'-9*). Remarkably, the branch sequences listed in PomBase contain the various nucleotides in the following proportions: in position 1 of the BS, C (77.2%), U (21.4%), A (1.1%), and G (0.3%); and in position 5 of the BS, C (69.3%), U (28.4%), and A (2.3%). Thus, at positions 1 and 5, C and U appear with the highest frequency, and both nucleotides can base-pair with G. Notably, the BSs GUXAG and AUXAA are not present in introns listed in PomBase.

Strikingly, the U at position 2 of the BS is 100% conserved. When this U was mutated to a G, the intron of the reporter gene was no longer recognized, regardless of the presence of inhibitor (Fig. 3B, *res1´-11*). In addition, U at position 2 is also essential for pre-mRNA splicing *in vitro* using budding yeast splicing extract. Most likely this U attracts the helicase spPrp11 (scPrp5/hsDDX46) that plays a role in selection of the BS [43, 44].

Next, we mutated the branchpoint A at position 4 to U (Fig. 3C, *res1´-12*). In this case, the intron was spliced but very inefficiently, even when Prp4 kinase was not inhibited. Addition of kinase inhibitor led to absolute intron retention. However, after 180 minutes of kinase inhibition, the mRNA was detected again, but splicing efficiency of the reporter gene remained very low.

Finally, we mutated position 3 in the BS, which is opposite the Ψ in snRNA U2 (Fig. 1A), from A to G, U, or C. Among the BSs listed in PomBase, 87.6% contain A at this position, 8.5% G, 2.8% U, and 1% C. The mutations of position 3 to U or C converted the Prp4-independent intron to a Prp4-dependent one, and pre-mRNA accumulation was observed immediately after inhibition of Prp4 kinase. After 180 minutes of inhibition, splicing resumed with high efficiency (Fig. 3D, *res1´-14* & *res1´-15*). Mutation of this position from A to G did not affect Prp4 dependency. The intron was spliced efficiently in the presence or absence of kinase inhibitor, i.e., it remained Prp4-independent (Fig. 3D, *res1´-13*). In conclusion, single mutations in the BS led in two cases to conversion from a Prp4-independent to a Prp4-dependent intron. When position 3 was U or a C, inhibition of Prp4 kinase resulted in typical splicing inhibition, followed by an inhibition reversion pattern [19] (Fig. 3D). In general, replacing a U with Ψ can enhance the thermodynamic stability of RNA duplexes. Moreover, Ψ can form hydrogen bonds with A, G, and U, of which Ψ-A and Ψ-G are the most thermodynamically stable. On the other hand, C cannot form hydrogen bonds with Ψ; consequently, this nucleotide has the least stable interaction with Ψ [45]. Based on our results, Prp4 kinase activity might facilitate the transient interaction between Ψ-U and Ψ-C.

In principal, Prp4 kinase activity appears to facilitate the efficient splicing of an intron when the 5´ SS or the BS cannot form stable transient complementary base-pairing with snRNAs U1 and U2, respectively. In this case Prp4 activity is involved in the recognition of weak 5´ SSs and BSs. Therefore, it is reasonable to probe as potential substrates of Prp4 kinase those proteins which are involved in intron recognition as discussed in the introduction. To test whether proteins involved during the first steps of intron recognition are substrates of Prp4 kinase we carried out *in vitro* kinase assays in the presence of $\gamma[^{32}P]$ ATP. As potential substrates we used purified proteins of snRNP U1 (spUsp101 (hsU1-70K), spUsp102 (hsU1A), spUsp103 (hsU1C) and spUsp107 (hsRBM25)), the three proteins which recognize the BS/3´ SS first (spBpb1 (hsSF1), spPrp2 (hsU2AF2) and spUaf2 (hsU2AF1)), the two SR proteins (spSrp1 (hsSRSF2) and spSrp2 (hsSRSF4/5/6)) and the SR-like protein spRsd1 (hsRBM39/23). Prp4 kinase phosphorylated spUsp101 and spBpb1 (Fig. 4A, lane 2 & 4B, lane 3) as well as the SR protein spSrp2 and the SR-like protein spRsd1 at high levels (Fig. 4B, lanes 5 & 6). The SR protein spSrp2 contains instead of a typical RS domain two RS elements, called RS1 and RS2, which are phosphorylated by spPrp4 kinase *in vitro*, whereas the typical RS domain in spSrp1 is not phosphorylated at all [7, 35] (Fig. 4). In contrast, phosphorylation of spUsp102, spUsp103, spUsp107 and spUaf2 was not detected. In case of spPrp2 phosphorylation by Prp4 kinase cannot be excluded. Both proteins have almost the same molecular weight and therefore we cannot separate

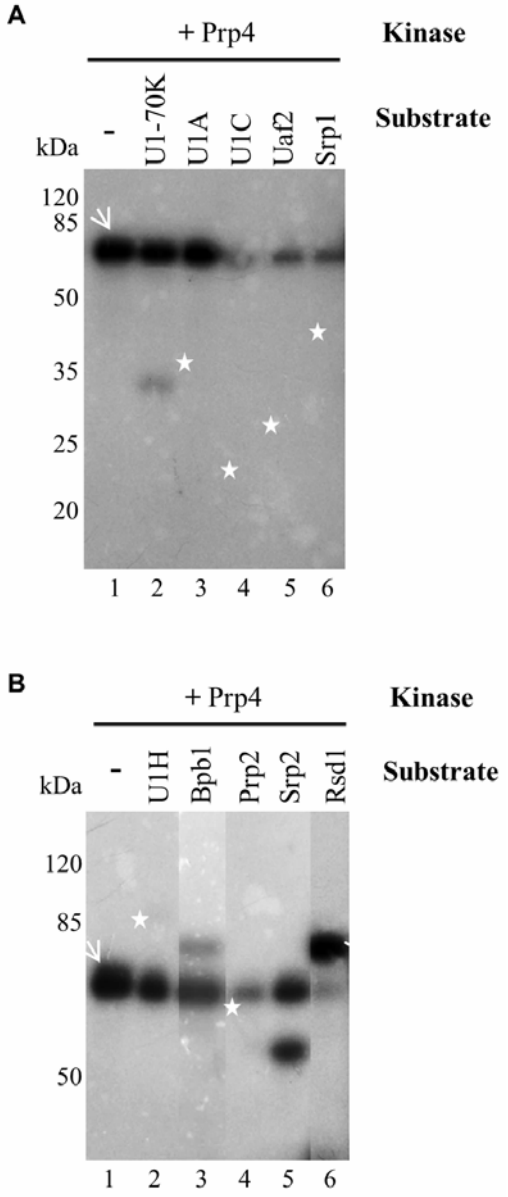

Fig. 4. Prp4 kinase phosphorylates spU1-70K, spBpb1, spSrp2 and spRsd1 *in vitro*. *In vitro* kinase assays were carried out with purified proteins in the presence of γ[^{32}P] ATP. Samples were resolved in (**A**) 12% or (**B**) 8% SDS polyacrylamid gels and visualized with X-ray film. Purified Prp4 kinase was individually incubated at 30 °C for 30 min with purified **(A)** spUsp101 (U1-70K, lane 2), spUsp102 (U1A, lane 3), spUsp103 (U1C, lane 4), spUaf2 (lane 5), spSrp1 (lane 6), **(B)** spUsp107 (U1H, lane 2), spBpb1 (lane 3), spPrp2 (lane 4), spSrp2 (lane 5), spRsd1 (lane 6). The autophosphorylation of Prp4 kinase is indicated with arrows. The expected positions of the phosphorylated proteins are indicated with asterisks. The numbers on the left side of the images show the sizes of protein bands (kDa).

the autophosphorylated Prp4 kinase from spPrp2 in the gel. The results show that Prp4 phosphorylates *in vitro* proteins involved in recognition of the 5´ SS and the BS/3´ SS as well as the SR protein spSrp2 and the SR-like protein spRsd1. Interestingly, a few years ago exon skipping mutants were identified in *S. pombe*. These mutants did not recognize the BS in the first intron and the 5´ SS in the second intron of the reporter gene. The genes isolated encode spBpb1 (hsSF1), spPrp2 (hsU2AF2) and spUaf2 (hsU2AF1) [46]. Moreover, recently it has been shown that overexpression of wild-type spSrp2 suppresses the phenotype of these three mutant alleles leading again to the recognition and splicing of both introns [47], thus demonstrating that spSrp2 can serve as a chaperone to facilitate 5´ SS and 3´ SS recognition.

CONCLUSION

We do not know yet how this facilitation operates molecularly. Based on our results it is conceivable that the interactions of spSrp2 with the 5´ exon/5´ SS and the 3´ region of the intron might be involved in the adjustment of the transient hybridization of snRNA U1 and snRNA U2 with the intron borders of weak SSs and BSs, respectively. It has been shown that spSrp2 interacts with spUaf2 which binds to the 3´ SS of the intron [48]. Based on the results of the *in vitro* kinase assays, we would not expect that spUaf2 is phosphorylated by Prp4 kinase. As also discussed previously, phosphorylated spSrp2 might be involved in presenting weak 5´ exon/5´ SS for proper complementary base pairing with snRNP U1 [19]. This structure has to be hold transiently in place during transcription and has to communicate and cooperate with the incoming snRNP U2. In mammalia the SR protein hsSRSF1 binds to the pre-mRNA and subsequent phosphorylation modulates its interaction with the protein hsU1-70K (spUsp101) [31]. Prp4 kinase phosphorylates spUsp101 (hsU1-70K) *in vitro* and therefore will be taken into consideration as a potential component in our suggested safeguarding system.

In case of the BS, where the branchpoint A is at position 4, it is comprehensible that this five nucleotide interaction with the indicated region in snRNA U2 is strongly dependent on appropriate

structure of the pre-mRNA and snRNA U2 (Fig. 1A). This structure can only be formed when spBpb1 is removed from the BS. Therefore, it is possible that phosphorylation of spBpb1 leads to a conformational change of the protein and subsequently to structural alteration of the pre-mRNA that allows the formation of these specific transient RNA duplexes between the BS and snRNA U2. This idea is supported by the splicing phenotypes of double mutations in the BS: when typical base-pairing interactions were abolished, the intron was either not recognized at all or required Prp4 kinase activity in order to be spliced (Fig. 3A). After the two RNA duplexes have been formed the system has to feed back that U1 and U2 are in the proper place on the intron. Although highly speculative this might be done by phosphorylation of spRsd1 which probably promotes the stable formation of the prespliceosomes [36]. Then the B-complex is established, the spliceosome is activated and the transesterification reactions occur.

We are aware that this model is highly speculative; however, taken all the previous and ongoing research with Prp4 kinase into consideration it outlines a way to think about it. Therefore, we propose that Prp4 kinase and its substrates are components of a safeguarding system that ensures intron recognition and safeguarded removal of introns with weak SSs and BSs, thereby keeping gene expression in synchronization with the life cycle of the cell under current environmental conditions. Incorrect gene expression might lead to failure of mitotic growth. Further identification and characterization of physiological substrates of Prp4 kinase will help to elucidate this intriguing system.

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AUTHORS’ CONTRIBUTIONS

D.E., N.A.B. and N.F.K. conceived the study and designed the experiments. S.Z.E., D.E. and N.A.B. performed the experiments and interpreted data. N.A.B., D.E. and N.F.K. analyzed data and wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

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