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Spliceostatin A inhibits spliceosome assembly subsequent to prespliceosome formation

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ABSTRACT

Pre-mRNA splicing is catalyzed by the large ribonucleoprotein spliceosome. Spliceosome assembly is a highly dynamic process in which the complex transitions through a number of intermediates. Recently, the potent anti-tumor compound Spliceostatin A (SSA) was shown to inhibit splicing and to interact with an essential component of the spliceosome, SF3b. However, it was unclear whether SSA directly impacts the spliceosome and, if so, by what mechanism, which limits interpretation of the drugs influence on splicing. Here, we report that SSA inhibits pre-mRNA splicing by interfering with the spliceosome subsequent to U2 snRNP addition. We demonstrate that SSA inhibition of spliceosome assembly requires ATP, key pre-mRNA splicing sequences and intact U1 and U2 snRNAs. Furthermore all five U snRNAs in addition to the SSA molecule associate with pre-mRNA during SSA inhibition. Kinetic analyses reveal that SSA impedes the A to B complex transition. Remarkably, our data imply that, in addition to its established function in early U2 snRNP recruitment, SF3b plays a role in later maturation of spliceosomes. This work establishes SSA as a powerful tool for dissecting the dynamics of spliceosomes in cells. In addition our data will inform the design of synthetic splicing modulator compounds for targeted anti-tumor treatment.

INTRODUCTION

Pre-mRNA splicing plays an essential role in the expression of eukaryotic genomes. Splicing removes intervening sequences (introns) that interrupt nearly all of human gene transcripts. Inefficiency or mistakes in splicing are implicated in an array of human diseases (1), and modulating splicing is proving to be an effective method to treat a collection of diseases (2–4). Cells have evolved an elaborate machine, known as the spliceosome, to carry out the two chemical steps of the splicing reaction at each intron. Spliceosomes assemble from key building blocks, which include five snRNAs (U1, U2, U4, U5 and U6) and their specific protein components (5). Together these components play critical roles in identifying conserved splicing signals in pre-mRNAs, assembling the catalytic spliceosome and facilitating splicing chemistry (6).

Spliceosome formation is very complex and has been modeled as a step-wise assembly through a number of intermediates (7). These intermediates (E, A, B and C complexes) were first identified by their migration in native gels and later defined by their U snRNP components. The first splicing complex, early complex (E complex), involves recognition of the 5′ splice site by U1 snRNA in an ATP-independent manner (8,9). At this point, U2 snRNP also appears to loosely associate with E complex (10,11). As E complex transitions to the prespliceosome (A complex), U2 snRNP is recruited to the branch point adenosine via interactions between the SF3b subcomplex protein SF3b1 and $U2AF^{65}$ (12). ATP-dependent stabilization of the interaction between U2 snRNP and the branch point sequence is a hallmark of A complex assembly (10). A fully assembled spliceosome (B complex) forms with the stable incorporation of the tri-snRNP (U4/U6•U5) and Prp19 complex. Following a series of ATP-dependent RNA:RNA and RNA:protein rearrangements and destabilization of U1 snRNP and U4 snRNP associations, the catalytic spliceosome (C complex) forms to carry out the two sequential transesterification reactions of splicing (13–15). In addition to the interactions noted above, there are many more intermolecular contacts among spliceosome components that remain to be determined. Furthermore, with the complexity of spliceosome dynamics, regulation and components, it is likely that many additional conformations of the splicing machinery exist to govern transitions between the known intermediate complexes.

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One challenge in studying splicing *in vivo* and *in vitro* is a lack of tools for arresting the spliceosome during its dynamic assembly pathway. Small molecule inhibitors of pre-mRNA splicing will be critical for manipulating spliceosomes to dissect their structure and function in the cell. A growing number of inhibitors of splicing and spliceosome assembly have been reported (3,4,16–27), however, the mechanism by which they exert their influence on splicing is unclear.

Spliceostatin A (SSA), originally identified as an anti-tumor compound (28), is a potent inhibitor of *in vitro* and *in vivo* pre-mRNA splicing (24). It has been suggested that SSA shares a pharmacophore structure with pladienolide, another splicing inhibitor (3). Both SSA and pladienolide have been shown to interact with the SF3b complex, which is a component of U2 snRNP and is essential for splicing (29,30). Although this finding suggests that SSA may directly interfere with U2 snRNP function, how the compound inhibits splicing remained to be determined. Before SSA can fulfill its promise as a general tool to manipulate splicing, it is necessary to understand the molecular mechanism of SSA-mediated splicing inhibition. Furthermore, because SSA, pladienolide and their derivatives have been shown to differentially inhibit a diverse collection of human tumor lines (28,31,32) understanding the molecular effects of these splicing modulators will be important for developing therapeutics that target-specific cancer types based on the compounds.

In the following study, we investigated the effects of SSA on the assembly of human spliceosomes. We find that the compound interferes with spliceosome assembly subsequent to stable U2 snRNP association with pre-mRNA. SSA inhibits spliceosome assembly by slowing the A to B complex transition, and the inhibition requires ATP, functional snRNPs and splicing signals in the pre-mRNA. During SSA inhibition all the U snRNAs as well as the SSA molecule associate with pre-mRNA. Collectively, our data suggest that SSA inhibits spliceosome assembly at the transition between A and B complex spliceosomes, and indicate a role for SF3b following U2 snRNP recruitment to the spliceosome. Our study opens the way towards examining spliceosome assembly at this important transition in cells.

METHODS

Plasmids and pre-mRNA *in vitro* transcription

For *in vitro* splicing reactions, we used a derivative of the adenovirus major late (AdML) transcript that contains three MS2 binding sites within the 3′ exon with an AG → GG 3′ splice site mutation (33) as a splicing substrate (10). To remove splicing consensus sequences, we mutated the 5′ splice site (GU → CU) and branch point (A → G) of this splicing substrate. Splicing substrates were generated by T7 runoff transcription, G(5′)ppp(5′)G-capped, body labeled with ^{32}P UTP and gel purified.

In vitro splicing reactions with SSA treated nuclear extract

HeLa nuclear extract was incubated with 5% MeOH, or indicated SSA concentrations for 1 h at 4°C. Subsequent *in vitro* splicing reactions consisted of 40% pre-treated nuclear extract, 2 nM pre-mRNA splicing substrate, 2 mM MgAc, 60 mM KGlu, 2 mM ATP, 5 mM creatine phosphate (CP) and 0.01 mg/ml yeast tRNA and were conducted at 30°C for times indicated. For ATP depletion, NE was pre-incubated at 30°C for 10 min and ATP and CP were omitted from the reactions. At indicated timepoints, RNA was isolated from splicing reaction by phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation and then separated by denaturing urea PAGE. Splicing gels were scanned using a Typhoon phosphorimager (Molecular Dynamics) and splicing efficiency calculated using Imagequant software.

Native gel analysis

To separate higher order complexes, splicing reactions were stopped at indicated time points by placing on ice and adding 5× native loading buffer (0.125 mg/ml heparin, 20 mM Tris, 20 mM glycine, 25% glycerol, 0.25% cyan blue, 0.25% bromophenol blue). After 5 min centrifugation at 4°C, samples were warmed to room temperature and loaded onto 2.1% native agarose gels. Gels were run in 20 mM Tris/glycine at 72 V for 3 h and 50 min. Gels used for complex analysis were immediately dried down on Whatman paper at 55°C for 30 min and exposed overnight before being analyzed using a phosphorimager.

To separate E and H complexes, 1.5% native agarose gels were used. In this case, heparin was not included with the ATP-depleted reactions untreated with SSA.

Complex pull downs

Pre-mRNA was bound with 50 molar excess of MS2-MBP and added to 1 ml splicing reactions. Following the indicated incubation conditions for each complex, the reactions were treated with 0.125 mg/ml heparin for 5 min at 30°C and then passed over 100 µl of amlyose resin three times at 4°C. The beads were washed extensively with 150 mM KCl, 2 mM $MgCl_2$ and 20 mM Tris pH 7.9. Complexes were eluted in the same buffer with 10 mM maltose. Control reactions included incubating splicing reactions in the absence of ATP for 30 min at 4°C, in the absence of ATP for 30 min at 30°C, or with NE pre-incubated with 2 µM of a 2′-*O*-methyl oligonucleotide complimentary to nucleotides 1–20 of U2 snRNA (34). SSA-inhibited spliceosomes were accumulated in splicing reactions containing NE treated with 200 nM SSA.

RNase H cleavage of U snRNAs

RNase H cleavage of U1 and U2 snRNAs was carried out as described in ref. (35) using the following DNA oligonucleotides: U1 5′-TTCAGGTAAGTACTCA-3′ (complementary to nucleotides 2–11) and U2 5′-CAGATACTAC ACTTG-3′ (complementary to nucleotides 28–42; 35). Digestions were verified by SYBR-Gold direct staining.

Streptavidin agarose resin pulldowns of biotinylated-SSA

NeutrAvidin agarose (Thermo Fisher Scientific) was blocked with 50× Denhardt's solution and washed with 150 mM KCl, 2 mM $MgCl_2$, 20 mM Tris pH 7.9. The beads were then incubated in splicing reactions containing 200 nM biotinylated SSA or non-biotinylated SSA and then washed with the same buffer. Total input and pre-mRNA in the unbound fraction, washes and bound fractions were measured using a Geiger counter. Pre-mRNA remaining on beads was quantified by comparing bound pre-mRNA c.p.m. counts to c.p.m. counts from total input, unbound fraction and wash fractions.

RESULTS

Spliceostatin A interferes with spliceosome assembly subsequent to A complex formation

SSA is an established inhibitor of splicing chemistry; however, whether the inhibition is due to an arrest of spliceosome assembly or to another indirect mechanism remained to be explored. Kaida *et al*. (24) identified the U2 snRNP subunit SF3b as a molecular target of SSA (24). SF3b complex plays an essential role in the ATP-dependent stable addition of U2 snRNP to E complex and it is critical for the assembly of prespliceosomes (36,37). We initially hypothesized that the binding of SSA to SF3b would interfere with U2 snRNP addition, leading to an arrest of spliceosome assembly at a point similar to E complex. To test this hypothesis we first confirmed that SSA inhibits splicing on our model substrate, a derivative of the adenovirus major late (AdML) pre-mRNA (13,38), by conducting *in vitro* splicing reactions in HeLa nuclear extract (NE) in the presence of increasing concentrations of SSA. It was previously reported that SSA blocks splicing before the first transesterification reaction (24). Similarly, relative to extracts treated with 5% MeOH, we observe <1% of splicing efficiency at the first step of chemistry when NE is treated with 200 nM SSA with our model substrate (Figure 1A).

To analyze SSA's effect on spliceosome assembly, we treated NE with SSA and used it for *in vitro* assembly of splicing complexes, which were then separated on 2.1% native agarose gels. These gels allow for the separation of the E→A→B→C spliceosome intermediates. With increasing concentrations of SSA, we clearly observe loss of higher order splicing complexes (Figure 1B). Surprisingly, in reactions treated with higher concentrations of SSA we observe accumulation of a pre-mRNA containing complex that migrates at a position near A complex spliceosomes (Figure 1B). Accumulation of this complex is proportional to the loss of higher order splicing complexes. At lower concentrations of SSA, B complex readily forms, but at concentrations of the drug that strongly inhibit splicing we see complete loss of B and C complexes. These data suggest that SSA inhibits assembly of catalytically competent spliceosomes subsequent to A complex formation.

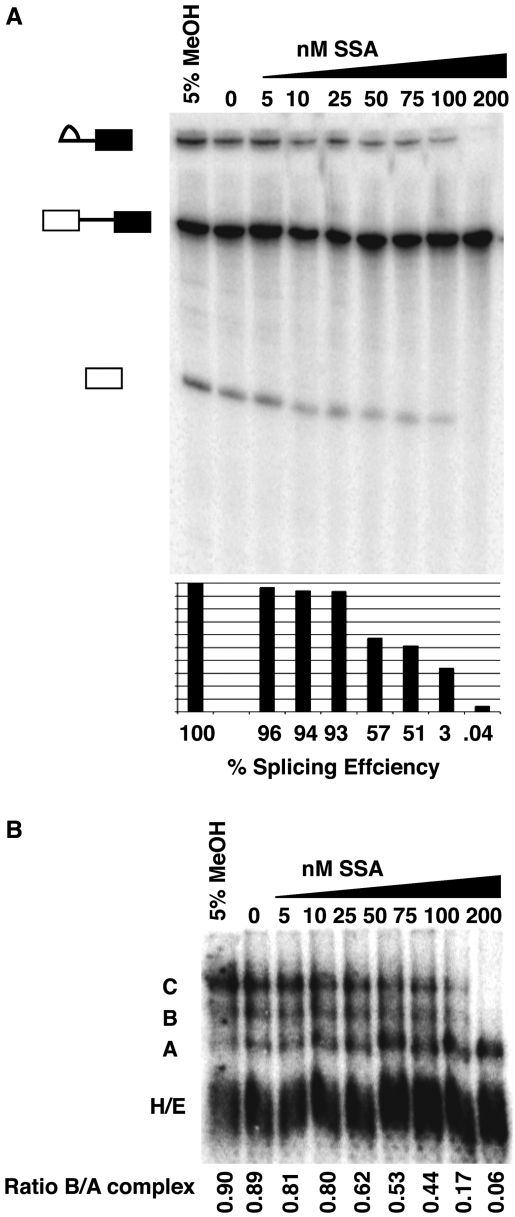

Figure 1. SSA inhibits spliceosome assembly: (**A**) denaturing PAGE analysis RNA from 25 min *in vitro* splicing reactions using an AdML splicing substrate in NE treated with indicated concentrations of SSA. We note that the substrate contains an AG → GG 3′ splice site mutation, which blocks splicing prior to exon ligation. We find that SSA does not impact the second step of splicing on wild-type substrates (Supplementary Data S1.) Splicing products schematized from top to bottom are lariat intermediate, pre-mRNA and mRNA. The total percent first step splicing activity (first step products/total RNA) indicated below each lane was normalized to 5% MeOH control splicing reaction. (**B**) Native agarose gel (2.1%) analysis of *in vitro* splicing reactions shown in (A). The 3′-splice site mutant accumulates C complex spliceosomes and allows better quantification of the different spliceosome assembly intermediate complexes. The position of C, B, A and H/E splicing complexes and SSA-inhibited spliceosomes are indicated. The ratio of B/A complex is indicated below each lane.

SSA inhibits spliceosome assembly following E complex formation

We next questioned whether the complex that forms on pre-mRNA in the presence of SSA represents an

intermediate in the spliceosome assembly pathway or a complex that forms independent of splicing. If the complex that accumulates with SSA treatment is a spliceosome intermediate, its migration in the native gels suggests that it is an A-like complex. A complex formation is preceded by E complex, but in the gels described above (2.1% native gels) we cannot differentiate E complex from H complex, which is the collection of proteins that associate with RNA in NE in the absence of splicing (39). To assess E complex formation in the presence of SSA, we analyzed a time course of spliceosome assembly on 1.5% native agarose gels, which separate E from H complex. In control reactions depleted of ATP, we observe the expected accumulation of E complex by 4 min. In reactions treated with SSA in the presence of ATP, E complex still assembles within 4 min. Moreover, E complex appears to progress to a stable higher order arrested complex by 15 min (Figure 2). These data are consistent with the complex that assembles in the presence of SSA being a spliceosome complex intermediate that transitions through E complex.

SSA inhibition of spliceosome assembly follows an ATP- dependent step

Following E complex, the next intermediate of normal spliceosome assembly is A complex, the formation of which requires ATP hydrolysis. Previous studies demonstrated that the stable addition of U2 snRNP underlies the ATP dependence of A complex assembly (12,36,37). If SSA inhibits spliceosome assembly following E complex, the observed drug-induced arrest of assembly

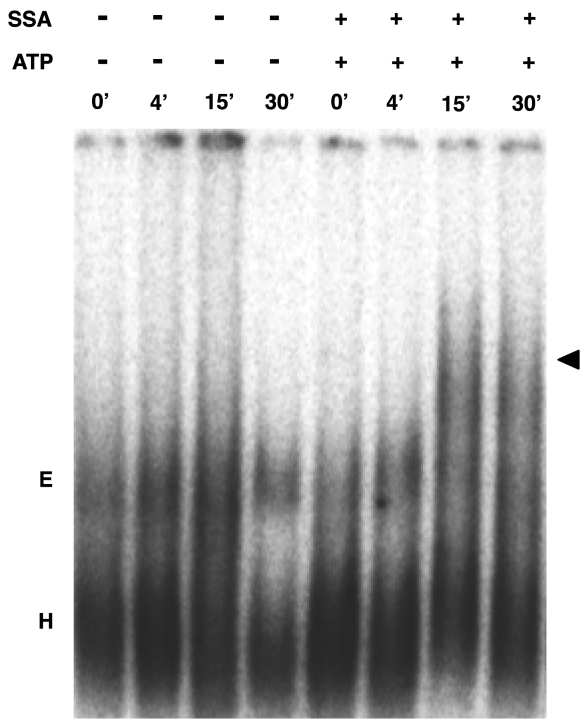

Figure 2. SSA inhibits spliceosome assembly following E complex formation: native agarose gel (1.5%) analysis of *in vitro* splicing reactions ±ATP and ±200 nM SSA.

should also exhibit a dependence on ATP. To address this issue, we carried out splicing reactions in NE treated with 200 nM SSA in the presence or absence of ATP and analyzed complex assembly on native agarose gels (Figure 3A). In contrast to ATP-containing reactions, we do not detect formation of the drug-inhibited complex in ATP-depleted reactions. This result implies that SSA inhibits spliceosome assembly subsequent to the ATP-dependent stabilization of U2 snRNP.

SSA inhibition of spliceosome assembly requires a 5′ splice site and branch point

Spliceosome assembly is guided by consensus sequences within the pre-mRNA substrate including 5′ splice site, 3′ splice site and branch point. If SSA is specifically inhibiting spliceosome assembly, formation of the drug-inhibited complex should be dependent upon conserved splicing signals. Figure 3A shows an SSA-inhibited complex forms on a substrate lacking a 3′ splice site. This result indicates that SSA inhibits spliceosome assembly before the 3′ splice site is required, which is during C complex formation with our model substrate. In contrast, when we carried out splicing reactions in the presence of SSA using a pre-mRNA substrates with mutant 5′ and 3′ splice sites or 5′ splice site mutation alone, the SSA-inhibited complex no longer forms (Figure 3B). Furthermore, the complex does not accumulate on a pre-mRNA with mutant branch point and 3′ splice site (Figure 3C). These data show that while the 3′ splice site is expendable for the assembly of the drug-inhibited complex, the 5′ splice site and branch point are necessary. From these results, we conclude that the observed drug-inhibited complex is likely a splicing complex because it has the same splicing sequence requirements as spliceosomes. We also conclude that SSA inhibits spliceosome assembly at a point following recognition of the 5′ splice site and branch point sequences. Given that the only demonstrated role for SF3b to date is in recognition of the branch point, this result also suggests that SSA may interfere with a subsequent function of SF3b in spliceosome assembly.

SSA inhibition of spliceosome assembly is dependent on functional U1 and U2 snRNPs

If the association of U snRNPs with pre-mRNA is relevant to SSA inhibition of splicing, then we expect formation of the drug-inhibited splicing complex to be dependent on U snRNP functionality. We looked at the roles of the U snRNPs by digesting key RNA sequences within U1 and U2 and assessed the subsequent effects on SSA inhibition by native gel analysis. We targeted regions in U1 and U2 snRNAs with DNA oligonucleotides to trigger RNase H cleavage, which is known to inhibit splicing (35). After verifying digest of specific snRNAs in nuclear extract (Supplementary Data S3), we used those extracts for spliceosome assembly with and without SSA treatment (Figure 3D). In untreated extracts in which U1 (ΔU1) or U2 (ΔU2) were digested, A complex spliceosomes failed to assemble. Likewise the drug-inhibited splicing complex forms much less efficiently

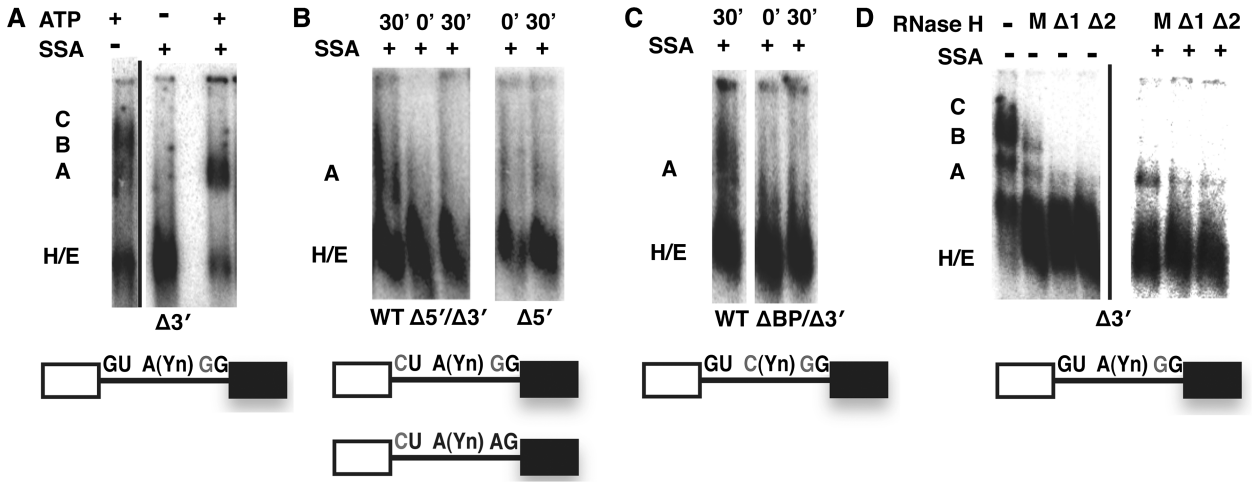

Figure 3. SSA inhibition of spliceosome assembly follows an ATP-dependent step and requires splicing signals and functional U1 and U2 snRNPs: (**A**) native agarose gel (2.1%) analysis of *in vitro* splicing reactions ±ATP and ±200 nM SSA as indicated. (**B**) Native agarose gel (2.1%) analysis of *in vitro* splicing reactions using pre-mRNA substrates that are wild-type (WT) or that contain both mutant 5′ and 3′ splice sites (Δ5′/Δ3′) or mutant 5′ splice site alone (Δ5′). Reactions were performed with NE treated with 200 nM SSA at the indicated time points. (**C**) Same as (B) but with a splicing substrate containing mutant branch point and 3′ splice site (ΔBP/Δ3′). (**D**) Native agarose gel (2.1%) analysis of 30 min time points of *in vitro* splicing reactions with nuclear extracts pre-incubated with DNA oligos complementary to U1 snRNA (ΔU1), U2 snRNA (ΔU2) or mock (M) for RNase H-mediated digestion of the targeted snRNA and ±200 nM SSA. Schematics of experimental splicing substrates are provided for each experimental condition.

after SSA treatment of either ΔU1 or ΔU2 extracts. These data indicate functional U1 and U2 snRNAs are required prior to SSA inhibition, likely in the same roles that they play in normal spliceosome assembly.

SSA slows the transition of A to B complex spliceosomes

We performed a kinetic analysis of spliceosome assembly at saturating and sub-saturating concentrations of SSA to better determine the assembly step at which SSA exerts its influence. In the absence of SSA the appearance of A complex by 1 min is followed by the formation of B complex, which begins to accumulate after 5 min. C complex is readily apparent after 15 min (Figure 4A). At saturating concentrations of SSA we observe only H/E complex at 0min, with SSA-inhibited spliceosomes accumulating by 4 min. We detect no progression to higher-order complexes. This result suggests that at saturating concentrations of SSA, progress to A complex may be slowed.

We conducted a time course at a sub-saturating concentration (75 nM) of SSA to more closely examine the stage(s) at which SSA affects spliceosome assembly. At this concentration, splicing is inhibited but not completely blocked. Under these conditions, we see that A complex forms with kinetics similar to untreated extracts, whereas the transition of A to B complex is slowed relative to no-drug condition. The rate of transition of B to C complex is largely unchanged (Figure 4B). We quantified the transition of A to B complexes in each of the conditions by determining the B/A ratio at each time point (Figure 4G). This analysis indicates a consistent slowing of the rate of A to B complex transition such that the limited assembly of B complex appears to be the primary consequence of SSA inhibition.

U1, U2 and tri-snRNP snRNAs associate with pre-mRNA during SSA inhibition of spliceosome assembly

Spliceosome assembly is modeled as an ordered addition of U snRNPs to pre-mRNA (7). Knowing the constellation of U snRNPs that associate with pre-mRNA in the presence of SSA will allow us to further define the mechanism of SSA inhibition of spliceosome assembly. We investigated the association of snRNAs with pre-mRNA during SSA inhibition by pull-down experiments. For the pull downs, we employed a pre-mRNA substrate bound by a fusion of MS2 coat protein and maltose binding protein (MS2:MBP). Following SSA inhibition of splicing with this substrate, we incubated the splicing reactions with amylose beads, washed extensively and eluted tagged pre-mRNA with maltose. To identify the U snRNAs associated with the drug-inhibited splicing complex, we isolated RNA from peak elution fractions, which was then separated in denaturing PAGE gels and stained with SYBR-Gold (Figure 5A). For comparison, we repeated pull downs from reactions in which we blocked spliceosome assembly by incubating splicing reactions on ice without ATP, at 30° without ATP or in the presence of a 2′-*O*-methyl oligonucleotide that hybridizes to the 5′-end of U2 snRNA (34; Figure 5A). We used reactions held at 4° with no ATP as a control for the background association of snRNAs to our pre-mRNA substrate. As expected in the absence of ATP at 30°, we observe U1 and U2 snRNAs in pre-mRNA pull downs (10). Previously, Lamond *et al.* (34) demonstrated that the U2-directed oligo blocks the transition of A to B complex spliceosomes, and when NE is treated with this oligo we observe association of U1 and U2 snRNAs with pre-mRNA. In contrast, in pre-mRNA pull downs from SSA-inhibited splicing reactions we see a very strong band for U2 as well as distinct bands for U1, U4, U5 and U6

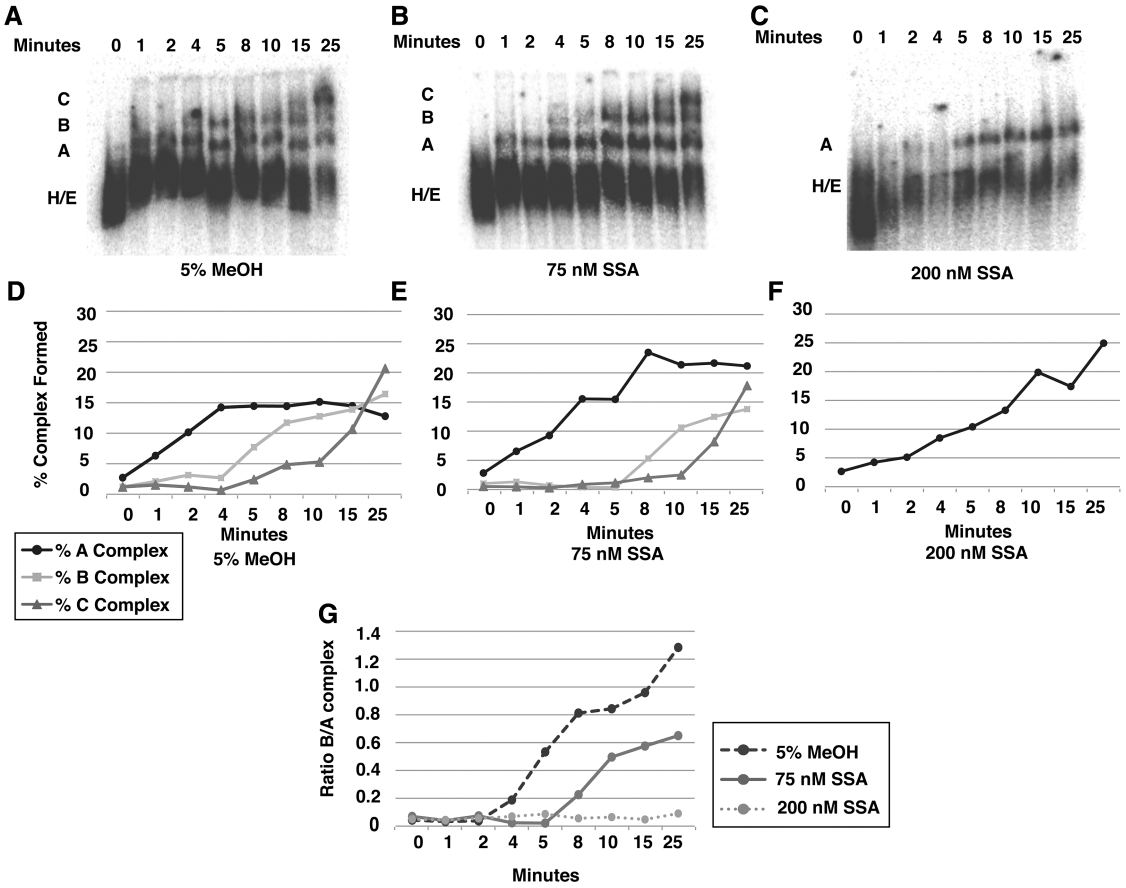

Figure 4. SSA slows the transition of A to B complex spliceosomes: 2.1% native agarose gels of time courses of spliceosome assembly in splicing reactions treated with either 5% MeOH (**A**), 75 nM SSA (**B**) or 200 nM SSA (**C**). (**D–F**) Relative amounts of A, B and C complexes were quantified from gels shown in (A, B and C), respectively and plotted versus time. (**G**) Ratio of B/A complex was quantified and plotted versus time for each condition tested.

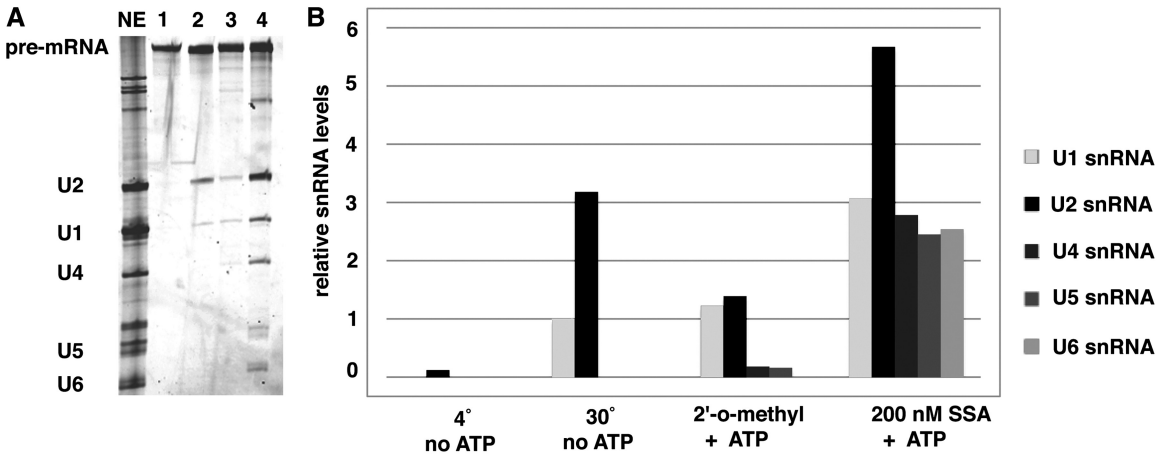

Figure 5. U snRNAs associate with pre-mRNA during SSA inhibition of spliceosome assembly. (**A**) SYBR-Gold direct staining of nucleic acid isolated from pull downs of spliceosomes assembled in NE treated under the following conditions: (i) 4°C, no ATP, (ii) 30°C, no ATP, (iii) anti-U2 2′ *O*-methyl oligonucleotide + ATP or (iv) 200 nM SSA + ATP. (**B**) Quantification of snRNAs detected within isolated spliceosome complexes normalized to pre-mRNA. We defined the pre-mRNA:U1 ratio as one for the 30° no-ATP pull down in order to compare between pull downs.

snRNA. In order to directly compare the association of snRNAs between the different samples, we normalized the amounts of each snRNA relative to pre-mRNA and defined the pre-mRNA:U1 ratio as one for the 30° no-ATP pull down (Figure 5B). The quantification confirms that the pre-mRNA pull down from SSA-inhibited reactions is enriched for all of the snRNAs relative to control preparations. We note that the

amounts of the U4, U5 and U6 snRNAs, while similar to each other, are relatively lower than those of U1 and U2. This observation may reflect a looser association of the tri-snRNP snRNAs with SSA-inhibited spliceosomes, such that a proportion of the tri-snRNAs are lost during washing of the beads. This supposition is supported by the recent findings that tri-snRNP initially associates with the spliceosome in an unstable manner before being stably integrated to form B complex (40,41). To confirm these results, we isolated RNA from the drug-inhibited complex band in native gels and see by northern analysis a similar enrichment of U2, U4, U5 and U6 snRNAs relative to the snRNAs present in an H/E band (Supplementary Data S2).

The interaction of SSA with pre-mRNA parallels U2 addition

Given that SSA does not interfere with U2 association with pre-mRNA and that the molecular target of SSA is the SF3b complex of U2 snRNP, it is possible that SSA is directly interfering with spliceosome assembly following U2 snRNP addition. If so, we predicted that SSA should be present in the drug-inhibited splicing complex. To investigate this possibility, we employed a biotinylated derivative of SSA (bio-SSA; 24). First we tested bio-SSA for its effect on *in vitro* splicing and spliceosome assembly. Although the *in vivo* activity of bio-SSA is ~20-fold weaker than SSA (24), we observe no measurable difference in its inhibition of *in vitro* splicing (Figure 6A). The weaker *in vivo* activity may be due to lower membrane permeability for bio-SSA. Notably, in the presence of bio-SSA, a complex identical to that observed with non-biotinylated SSA accumulates (data not shown).

We tested whether SSA associates with pre-mRNA during SSA inhibition by inhibiting splicing with bio-SSA and then incubating the reactions with streptavidin beads. After washing the beads, we compared the amount of pre-mRNA-bound streptavidin beads from reactions containing non-biotinylated SSA and see a significant and reproducible increase in pre-mRNA binding from reactions with bio-SSA (Figure 6B). Notably, the amount of pre-mRNA bound is 2-fold higher when ATP is included in the assembly reaction. In contrast, when we repeated the pull-down experiment with a pre-mRNA containing a mutated branch point in the presence of ATP, we see no enrichment of pre-mRNA with bio-SSA versus non-biotinylated SSA. These data support that SSA is present within SSA-inhibited spliceosomes and that its stable association with pre-mRNA is an ATP- and branchpoint-dependent event, like its U2 snRNP target.

DISCUSSION

SSA is one of a limited number of established inhibitors of pre-mRNA splicing reported (3,4,16–27). SSA is the only compound to work in the low nanomolar range and holds great promise as a tool for studying splicing *in vitro* and *in vivo*. Kaida *et al.* (24) previously demonstrated that SSA binds the U2 snRNP SF3b complex, making it one of the first small molecules known to target a spliceosome component. Understanding the mechanism of SSA inhibition of splicing is fundamentally important for accurately interpreting experiments using SSA to manipulate the splicing process. Furthermore, because SSA represents an exciting lead compound for the development of

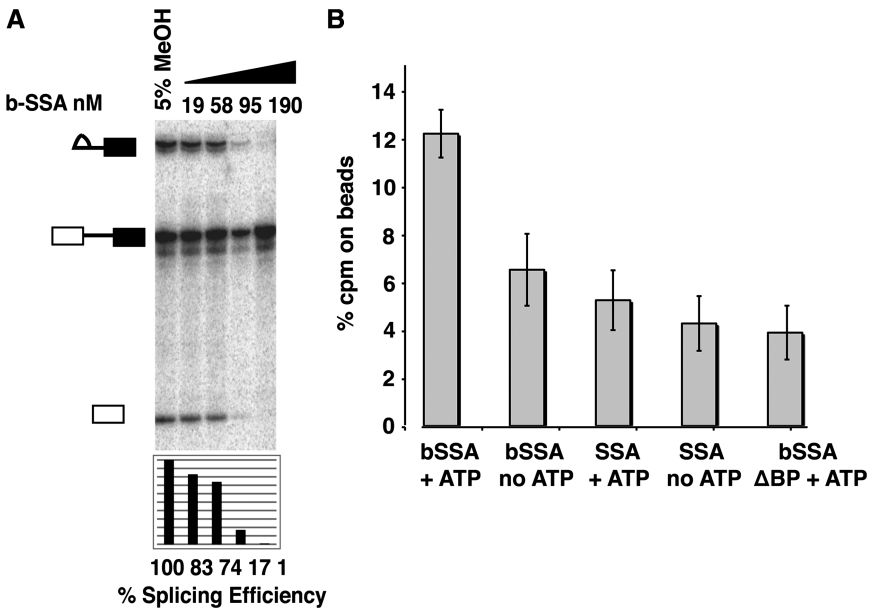

Figure 6. Biotinylated SSA (b-SSA) associates with pre-mRNA in an ATP-dependent manner. (**A**) Denaturing PAGE analysis of *in vitro* splicing reactions using an AdML splicing substrate containing a 3′ splice site mutation with increasing b-SSA and quantified as in Figure 1A. (**B**) Streptavidin agarose bead pull downs of pre-mRNA from *in vitro* splicing reactions containing 200 nM SSA or bio-SSA ± ATP. Pre-mRNA binding is indicated as the average percentage c.p.m. of total input radioactivity retained on beads from triplicate experiments. ΔBP is the same pre-mRNA except containing a branch point mutation.

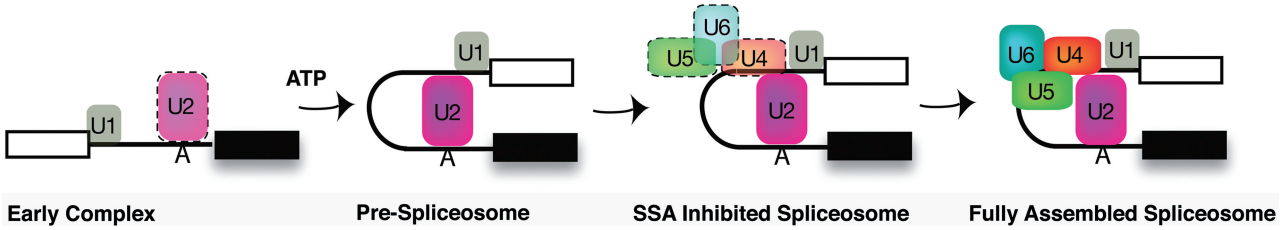

Figure 7. Model of SSA-mediated inhibition of spliceosome assembly: solid shapes denote stable interactions, whereas dotted/lighter colored shapes denote less stable association.

anti-tumor drugs, a molecular understanding of SSA action will certainly inform future studies concerning SSA derivatives and their potential as cancer therapeutics.

This report provides a detailed description of the kinetics, associations and dependencies of the effect of SSA on splicing and illuminates the mechanism by which SSA inhibits the spliceosome. One of our conclusions is that that SSA directly inhibits spliceosome assembly. In the presence of SSA, a complex that shares the same requirements as spliceosomes forms on pre-mRNA. SSA-inhibited spliceosomes do not form in the absence of a 5′ splice site, branch point adenosine or ATP and require functional U1 and U2 snRNAs (Figure 7).

Second, the data show that SSA does not inhibit splicing by blocking U2 snRNP association with pre-mRNA. In fact, we see more U2 snRNA associate with pre-mRNA in SSA inhibited reactions relative to control preparations. These data suggest that SSA may lead to a hyper-stabilization of U2 snRNP. In combination with the previously noted dependencies, our data show that SSA directly interferes with spliceosome maturation after U2 snRNP has stably integrated into the complex.

Finally, we conclude that SSA interferes with the transition of prespliceosomes to B complex. Our kinetic data reveal that while formation of pre-spliceosomes is somewhat slowed at high concentrations of SSA, at sub-saturating concentrations, SSA most strongly impacts formation of fully assembled B complex. This conclusion is also supported by the apparent accumulation of an A-like complex in the presence of SSA. The pre-spliceosome to B complex transition is hallmarked by the recruitment and stable incorporation of the tri-snRNP to pre-spliceosomes (42,43). We see that tri-snRNP interacts with the drug-inhibited complexes, but because those complexes do not migrate as a stable B complex in native gels, our data suggest that while SSA does not prevent association of tri-snRNP with the spliceosome, it may interfere with its stable integration.

Given that SSA targets SF3b, it is reasonable to suspect that its inhibition of splicing is mediated through SF3b. SF3b has been shown to interact with the branch point adenosine and to be required for the recruitment of U2 snRNP to the spliceosome (12). Our data argue that SSA does not interfere with this established function in U2 snRNP recruitment to the spliceosome. Instead the data suggest that SF3b may participate in an important interaction and/or promote a conformation necessary for the transition of A to B complex spliceosomes. The fact that SF3b has been shown to crosslink to pre-mRNA in B and C complex spliceosomes supports this potential role (12). Future studies to characterize the composition, intra-molecular interactions and structure of the splicing complex that accumulates in the presence of SSA may provide insight into this poorly understood transition and into the role of the SF3b subunit following U2 snRNP recruitment to the spliceosome.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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