

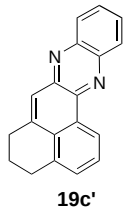
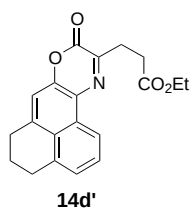
Table 5. Data for selected compounds: **1**, **2a**, **3a**, and **8**.

**1**: Colorless needles; m.p. 112–113 °C (hexane/Et<sub>2</sub>O 2/1);  $R_f$  = 0.35 (silica gel, hexane/EtOAc 1/1); IR (film):  $\tilde{\nu}_{\max}$  = 3378, 2967, 2931, 2867, 1664, 1506, 1463, 1427, 1399, 1239, 1180, 1081, 1055, 754 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  = 7.20 (br s, D<sub>2</sub>O exchangeable, 1H), 5.85 (t,  $J$  = 2.2 Hz, 1H), 5.66–5.61 (m, 1H), 5.46 (ddd,  $J_1$  = 9.9 Hz,  $J_2$  = 4.1 Hz,  $J_3$  = 1.2 Hz, 1H), 4.82 (br s, D<sub>2</sub>O exchangeable, 1H), 3.26 (ddd,  $J_1$  = 11.0 Hz,  $J_2$  = 7.0 Hz,  $J_3$  = 4.0 Hz, 1H), 3.23–3.18 (m, 1H), 2.73–2.66 (m, 1H), 2.50–2.30 (m, 1H), 2.45 (sep,  $J$  = 7.0 Hz, 1H), 2.35–2.36 (m, 1H), 1.95–1.82 (m, 2H), 1.75–1.61 (m, 2H), 1.57–1.48 (m, 1H), 1.44–1.31 (m, 1H), 1.18 (d,  $J$  = 7.0 Hz, 3H), 1.17 (d,  $J$  = 7.0 Hz, 3H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>):  $\delta$  = 191.8, 179.5, 167.9, 131.2, 126.3, 121.1, 84.0, 40.4, 36.7, 35.9, 34.7 (2 C), 30.5, 23.2, 20.4, 19.7, 19.0; HR-MS (MALDI-FTMS) calcd for C<sub>17</sub>H<sub>23</sub>NO<sub>3</sub>Na [ $M$ +Na<sup>+</sup>]: 312.1570, found: 312.1573

**2a**: Colorless plates; m.p. 206–208 °C (hexane/CH<sub>2</sub>Cl<sub>2</sub>);  $R_f$  = 0.29 (silica gel, hexane/EtOAc 1/1); IR (film):  $\tilde{\nu}_{\max}$  = 3347, 2919, 2847, 1655, 1592, 1519, 1477, 1452, 1384, 1332, 1241, 1159, 1123, 1093, 1027, 787 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  = 6.77 (s, 1H), 6.67 (bd,  $J$  = 7.9 Hz, 1H), 6.47 (bd,  $J$  = 7.9 Hz, 1H), 6.15 (br s, 1H), 6.09 (br s, D<sub>2</sub>O exchangeable, 1H), 5.82–5.75 (m, 2H), 5.24 (br s, D<sub>2</sub>O exchangeable, 1H), 4.70 (br s, D<sub>2</sub>O exchangeable, 1H), 3.21 (dt,  $J_1$  = 20.2 Hz,  $J_2$  = 4.4 Hz, 1H), 3.10–3.00 (m, 1H), 2.96 (dd,  $J_1$  = 20.2 Hz,  $J_2$  = 8.3 Hz, 1H), 2.87–2.75 (m, 2H), 2.19–2.11 (m, 1H), 2.10 (s, 3H), 2.00–1.82 (m, 2H), 1.51–1.40 (m, 1H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 152.7, 141.7, 137.1, 135.5, 133.9, 132.4, 132.2, 129.0, 124.6, 123.2, 120.2, 115.3, 114.9, 112.8, 35.2, 30.4, 29.5, 26.6, 22.9, 21.0; HR-MS (MALDI-FTMS) calcd for C<sub>20</sub>H<sub>21</sub>NO<sub>2</sub> 308.1645 [ $M$ +H<sup>+</sup>], found: 308.1646

**3a**: colorless cubes; m.p. 142–143 °C (hexanes/Et<sub>2</sub>O);  $R_f$  = 0.49 (silica gel, hexane/EtOAc 1/1); [ $\alpha$ ]<sub>D</sub> = +139.7° (0.35, CHCl<sub>3</sub>); IR (film)  $\tilde{\nu}_{\max}$  = 3283, 3017, 2929, 2862, 1740, 1678, 1437, 1374, 1239, 1202, 1147, 1086, 786 cm<sup>-1</sup>; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 5.88 (t,  $J$  = 2.3 Hz, 1H), 5.71–5.63 (m, 1H), 5.46 (dq,  $J_1$  = 10.0 Hz,  $J_2$  = 2.0 Hz, 1H), 4.11 (br s, D<sub>2</sub>O exchangeable, 1H), 3.77 (s, 3H), 3.58 (d,  $J$  = 8.6 Hz, 1H), 3.23 (br s, D<sub>2</sub>O exchangeable, 1H), 3.80 (s, 3H), 3.74–3.63 (m, 1H), 3.46 (d,  $J$  = 9.0 Hz, 1H), 3.15–3.08 (m, 1H), 2.73–2.64 (m, 1H), 2.47 (bd,  $J$  = 15.5 Hz, 1H), 2.38–2.11 (m, 2H), 2.20–2.10 (m, 1H), 1.84 (dt,  $J$  = 13.2, 2.2 Hz, 1H), 1.75–1.53 (m, 5H), 1.46–1.36 (m, 1H), 1.35 (d,  $J$  = 5.9 Hz, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 194.2, 171.3, 167.8, 131.7, 127.6, 123.1, 96.8, 76.9, 66.5, 52.8, 42.6, 36.8, 34.9 (2 C), 30.7, 23.4, 20.5, 18.5; HR-MS (MALDI-FTMS) calcd for C<sub>18</sub>H<sub>23</sub>NO<sub>4</sub> [ $M$ +H<sup>+</sup>]: 318.1700, found: 318.1698

**8**: Yellow oil;  $R_f$  = 0.51 (silica gel, hexane/EtOAc 2/1); IR (film):  $\tilde{\nu}_{\max}$  = 3330, 3028, 2969, 2932, 2873, 1688, 1668, 1613, 1504, 1467, 1385, 1318, 1157, 1099, 1026, 944, 884 cm<sup>-1</sup>; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.89 (br s, D<sub>2</sub>O exchangeable, 1H), 7.39 (s, 1H), 6.02 (dq,  $J_1$  = 9.9 Hz,  $J_2$  = 2.0 Hz, 1H), 5.47 (dq,  $J_1$  = 9.9 Hz,  $J_2$  = 3.2 Hz, 1H), 3.08 (dd,  $J_1$  = 9.8 Hz,  $J_2$  = 8.3 Hz, 1H), 2.60–2.49 (m, 2H), 2.25–2.13 (m, 3H), 2.00 (dd,  $J_1$  = 12.9 Hz,  $J_2$  = 8.5 Hz, 1H), 1.88–1.59 (m, 4H), 1.22 (d,  $J$  = 6.9 Hz, 6H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 202.2, 198.3, 177.5, 138.3, 131.0, 124.0, 118.5, 59.7, 53.4, 45.7, 37.1, 35.8, 30.0, 26.0, 21.5, 19.4, 19.3; HR-MS (MALDI-FTMS) calcd. for C<sub>17</sub>H<sub>21</sub>NO<sub>3</sub> [ $M$ +H<sup>+</sup>]: 288.1594, found: 288.1589



product like compounds are now readily accessible. A solid-phase version that uses ketohydroxyamides in a “heterocycle release” strategy<sup>[6]</sup> may expand the scope of the reported chemistry. Biological screening of the synthesized and projected compound libraries is expected to facilitate chemical biology studies and pharmaceutical research.

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## Separation of Spliceosome Assembly from Catalysis with Caged pre-mRNA Substrates\*\*

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Pre-messenger RNAs (pre-mRNAs) in eukaryotes are characterized by a split-gene structure in which coding exon sequences are separated by noncoding intron sequences.<sup>[1]</sup> The process by which the introns are excised from the pre-mRNA and the exons are joined together is known as pre-mRNA splicing and is catalyzed by the spliceosome—a biochemical machine that contains both protein and RNA components.<sup>[2]</sup> The spliceosome includes the U1, U2, and U4/

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U5/U6 snRNPs (small nuclear ribonucleoprotein particles), each containing a unique RNA and associated proteins. In contrast to many biocatalysts, the functional spliceosome does not appear to exist independently of its substrate; instead, the active splicing catalyst assembles in an ATP-dependent, stepwise, ordered fashion on pre-mRNA substrates through the A, B, and C complexes, which may be visualized by native gel electrophoresis (Figure 1a).<sup>[3]</sup> It would clearly be useful to separate spliceosome assembly from the subsequent chemical steps of pre-mRNA splicing, such that the requirements for each could be studied independently.

The chemistry of pre-mRNA splicing involves two sequential transesterification reactions. During the course of spliceosome assembly, a specific adenosine residue in the branch region of the intron bulges out from an RNA duplex formed between the pre-mRNA and the U2 snRNA (Figure 1b). The 2'-hydroxy group of this residue carries out a nucleophilic attack at the 5'-splice site to generate a free 5'-exon and a cyclic intermediate containing a 2'-5'-phosphodiester branch.<sup>[4]</sup> Attack of the free 5'-exon at the 3'-splice site then yields ligated exons and a cyclic intron product. Removal of the nucleophilic hydroxy group from the branch adenosine by placement of a 2'-deoxy residue at this position prevents the branch formation from occurring at this position.<sup>[5]</sup>

Here we describe an approach to the study of pre-mRNA splicing whereby reactivity of the branch adenosine in a pre-mRNA is transiently blocked with a photolabile caging group. Despite this modification to the pre-mRNA, the spliceosome is able to assemble on the caged substrate; subsequent photolysis frees the branch nucleophile to initiate the two transesterification reactions of splicing. Thus a caging approach effectively separates spliceosome assembly from catalysis.

We have previously demonstrated in the hammerhead ribozyme system that the reactivity of a unique 2'-hydroxy group within RNA can be transiently blocked by the presence of a photolabile nitrobenzyl ether at this position.<sup>[6]</sup> In order

to prepare a caged pre-mRNA, we chemically synthesized a ten-base oligomer in which the branch adenosine was modified at the 2'-position as an *ortho*-nitrobenzyl ether<sup>[6-8]</sup> and all other 2'-positions were protected with acid-labile 1-(2-fluorophenyl)-4-methoxypiperidin-1-yl (Fpmp) groups. Following standard workup, the purified caged oligomer was ligated to products of T7 RNA polymerase transcription, representing the 5'- and 3'-portions of the PIP85.B pre-mRNA substrate, in the presence of a bridging DNA oligonucleotide to yield a full length pre-mRNA with a caged branch nucleotide (Figure 2a).<sup>[9, 10]</sup> The resulting RNA contained a single <sup>32</sup>P-radiolabel situated two nucleotides in the 3'-direction of the caged nucleotide, which would facilitate analysis by denaturing polyacrylamide gel electrophoresis (PAGE).

We examined the reactivity of the caged RNA by carrying out photolysis experiments, with a xenon arc lamp as a light source, followed by RNase A and RNase T1 digestion and thin layer chromatography (TLC) analysis of the resulting RNA fragments. Although control RNAs were unaffected by photolysis, 90% of the caging groups were removed upon irradiation to yield free 2'-hydroxy groups (Figure 2b, c).

We tested the behavior of the caged pre-mRNA under splicing conditions by incubating it in HeLa cell nuclear extract<sup>[11]</sup> in the presence of adenosine 5'-triphosphate (ATP) and Mg<sup>2+</sup>—both of which are required cofactors for pre-mRNA splicing. Products associated with the first and second transesterification reactions of splicing are typically detected after an approximately 20 minute lag time required for the assembly of the spliceosome. However, in the case of the caged pre-mRNA, a negligible amount of splicing product was observed (approximately 3%, due to some uncaging during handling; Figure 3b). The formation of spliceosomes on the caged pre-mRNA was confirmed by nondenaturing PAGE (Figure 3a).<sup>[4]</sup> Thus, while the caging group blocks the first chemical step of splicing, the caged

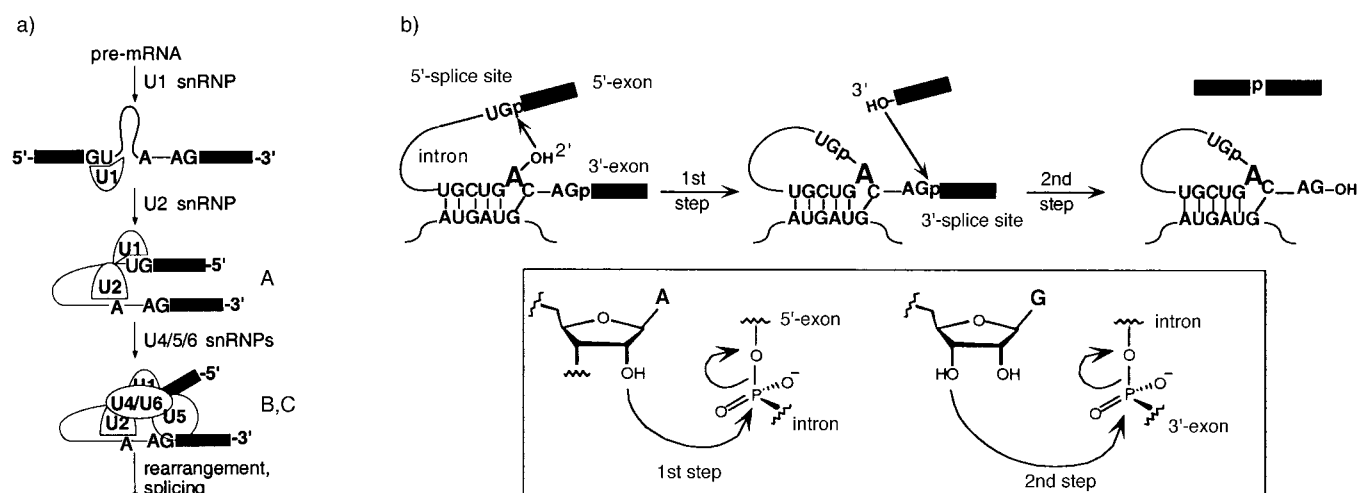


Figure 1. a) Assembly of individual snRNP particles on a pre-mRNA to form the active spliceosome. The conserved 5'-splice site (GU), branch site (A), and 3'-splice site (AG) sequences on the pre-mRNA are shown. The complexes formed as the spliceosome assembles are referred to as the A, B, and C complexes. b) Sequential transesterification reactions catalyzed by the spliceosome. In the first step, the 2'-hydroxy group of the branch adenosine (A) within the intron carries out a nucleophilic displacement at the 5'-splice site to generate a free 5'-exon and a cyclic intermediate. Attack of the free 5'-exon at the 3'-splice site then yields ligated exons and a cyclic intron product.

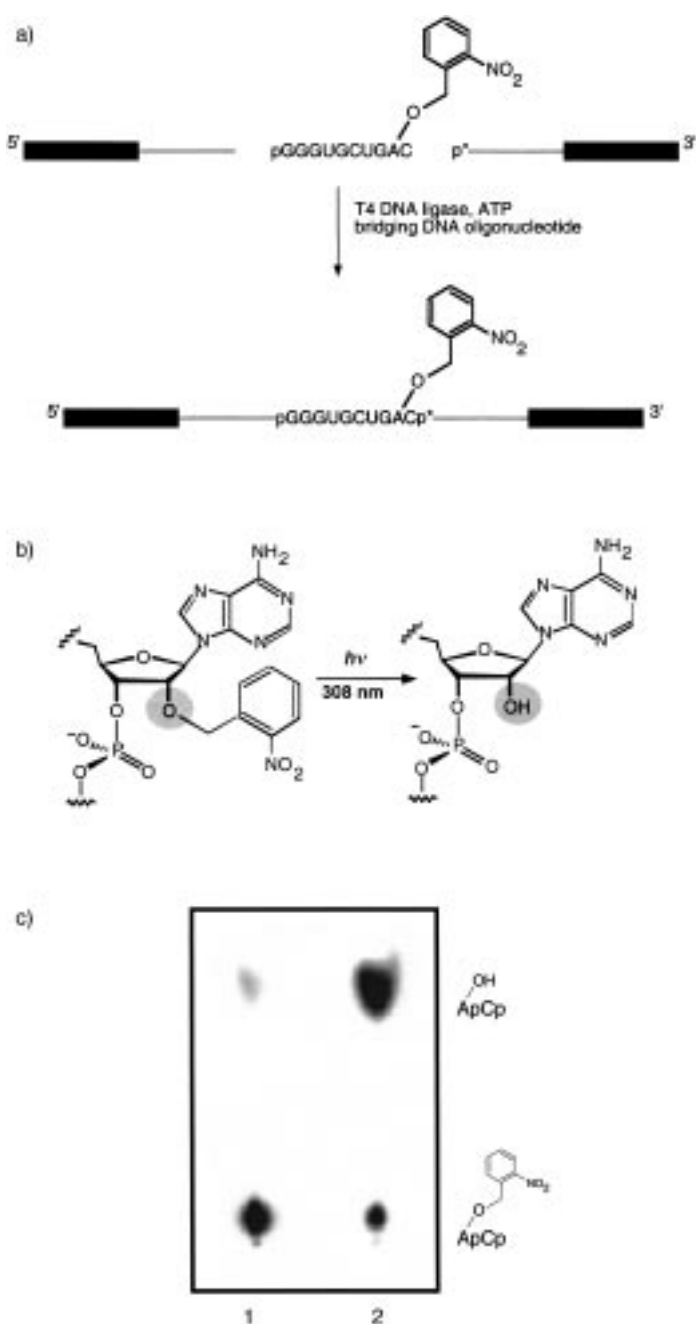


Figure 2. a) Synthesis of pre-mRNAs containing a caged 2'-hydroxy group at the branch position. A synthetic oligomer with a single modified adenosine residue was ligated to T7 RNA polymerase transcripts to yield full-length caged pre-mRNA with a single  $^{32}\text{P}$ -radiolabel (shown as p\*). b) Photolysis of caged adenosine to yield free adenosine. c) TLC analysis of photolysis of the caged pre-mRNA. Lane 1: unphotolyzed, caged pre-mRNA digested with RNase A and RNase T1; lane 2: photolyzed, uncaged pre-mRNA digested with RNase A and RNase T1.

pre-mRNA is still able to direct formation of the spliceosome.

In order to determine whether the spliceosomes formed on the caged substrates were catalytically competent, we incubated caged pre-mRNA in HeLa cell nuclear extract under splicing conditions, photolyzed the resulting complexes, removed aliquots over time, and analyzed the RNA by denaturing PAGE. These experiments showed that uncaging

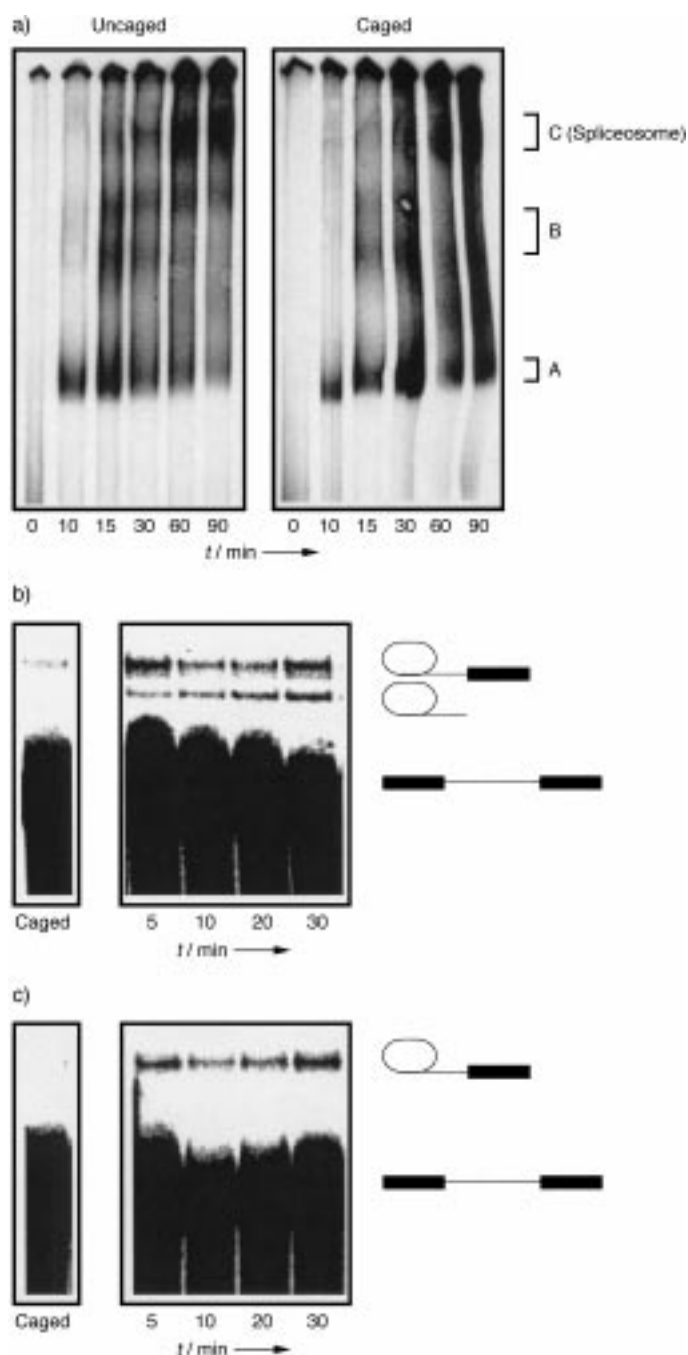


Figure 3. a) Spliceosome formation on uncaged and caged pre-mRNAs. Uncaged and caged  $^{32}\text{P}$ -labeled pre-mRNAs were incubated under splicing conditions in HeLa cell nuclear extract for the indicated times, to allow formation of the A, B, and C (spliceosome) complexes, and analyzed by native gel electrophoresis (4%; 80:1; 50 mM trisglycine). b) Uncaging of pre-mRNA in HeLa cell nuclear extract; denaturing PAGE analysis of  $^{32}\text{P}$ -labeled RNA. Left: caged pre-mRNA incubated for 60 minutes under splicing conditions; right: time course of splicing, after photolysis of pre-mRNA · spliceosome complexes, showing production of cyclic intermediate (first chemical step) and cyclic intron (second chemical step) products. c) Cofactor requirements after assembly of the spliceosome; denaturing PAGE analysis of  $^{32}\text{P}$ -labeled RNA. Caged pre-mRNA was incubated in HeLa cell nuclear extract under splicing conditions to form pre-mRNA · spliceosome complexes after which ATP was depleted from the reaction by the addition of glucose and hexokinase. Left: caged pre-mRNA incubated for 60 minutes under splicing conditions; right: time course of splicing, after ATP depletion and photolysis of pre-mRNA · spliceosome complexes, showing production of cyclic intermediate (first chemical step) but no cyclic intron (second chemical step) products.

of the pre-mRNA occurred cleanly within the spliceosome and furthermore that the two chemical steps of splicing proceeded without a delay, which is consistent with the fact that the caged RNA is sequestered within a fully formed spliceosome (Figure 3b).

We examined the cofactor requirements of splicing by incubating caged pre-mRNA in HeLa cell nuclear extract in the presence of ATP to allow the ATP-dependent formation of the spliceosome. Following a 60 minute period, the reaction mixture was depleted of ATP by addition of glucose and the enzyme hexokinase.<sup>[12]</sup> The reactions were irradiated to uncage the adenosine nucleophile and the products were analyzed by denaturing PAGE. Although the first step of splicing was observed under these conditions, depletion of ATP had the effect of blocking the second step (Figure 3c). Addition of excess ATP resulted in complete recovery of the second step of splicing in these reactions (data not shown).

Although ATP is required for spliceosome assembly, depletion of ATP from reactions containing fully formed spliceosomes did not prevent the first-step chemistry. This suggests that the caged pre-mRNA was poised to perform the first transesterification step and further indicates that ATP hydrolysis is in no way coupled to the nucleophilic attack at the 5'-splice site (the first step). ATP depletion did prevent the second step of splicing, which is in agreement with genetic studies in yeast<sup>[13]</sup> and also with the ATP requirement for second-step chemistry in a trans-splicing system.<sup>[14]</sup> This requirement most likely reflects the ATP requirement of RNA helicases involved in the rearrangement of RNA structure for the second step.<sup>[2]</sup>

Our preliminary investigations show that spliceosome assembly can be uncoupled from the chemistry catalyzed by the spliceosome with caged pre-mRNAs. This approach should prove valuable in the elucidation of cofactor requirements, both during the assembly of the spliceosome and between the two chemical steps, and in the investigation of the possible role of kinase and phosphatase activities in pre-mRNA splicing.<sup>[15]</sup> Finally the caging approach may be useful in transiently blocking reactivity in other RNA systems, including the Group II self-splicing intron.

### Experimental Section

**Preparation of caged pre-mRNA:** A ten-base oligoribonucleotide containing a single caged adenosine was synthesized and purified as described elsewhere.<sup>[6]</sup> The full-length caged pre-mRNA was synthesized by incubating a 5'-phosphorylated synthetic branch oligomer (300 pmol) with upstream (300 pmol) and 5'-<sup>32</sup>P-labeled downstream (100 pmol) T7 RNA transcription products (representing the 5'- and 3'-portions of the PIP85.B pre-mRNA<sup>[5]</sup>) in the presence of a bridging DNA oligonucleotide and 40 units of T4 DNA ligase (Conditions: 60 mM tris(hydroxymethyl)amino-methane (Tris) buffer (pH 7.8), 20 mM MgCl<sub>2</sub>, 36 units of ribonuclease inhibitor, 1.2 mM ATP, 2.4% polyvinylpyrrolidone (PVP-40), 5 mM 1,4-dithiothreitol) at 30 °C for 3 hours. One unit is the quantity of enzyme required to transform one nmol of substrate in twenty minutes at 37 °C. Ligations were purified directly by PAGE (15%; 29:1). The products were visualized by autoradiography, extracted, dissolved in doubly distilled water, and stored at -20 °C.

**TLC analysis of pre-mRNA:** Caged pre-mRNAs containing a single <sup>32</sup>P-label situated two nucleotides in the 3'-direction of the modification were digested with 2 units each of RNase T1 and RNase A (20-μL reaction volume, 10 mM Tris buffer (pH 7)) both before and after photolysis

(irradiation at a distance of 1 cm with a 1000 W Oriel Xenon arc lamp for four seconds in 5 mm pyrex reaction vessels). Reaction mixtures were then concentrated, loaded onto a cellulose polyethyleneimine TLC plate (4 × 10 cm, J. T. Baker), and eluted for 1 hour in saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>/1M NH<sub>4</sub>OAc/2-propanol (79:19:1). The air-dried TLC plate was exposed to a Molecular Dynamics phosphor screen and then scanned using a Molecular Dynamics Storm 860 Phosphorimager.

**Splicing and photolysis of caged pre-mRNA:** RNAs (50–100 × 10<sup>3</sup> cpm) were incubated in 10-μL reaction mixtures containing 40% HeLa cell nuclear extract, 2 mM MgCl<sub>2</sub>, 20 mM KCl, 1 mM ATP, 5 mM creatine phosphate, 4 units of ribonuclease inhibitor, and 4 μg of tRNA. Following a 60 minute incubation period at 30 °C, splicing complexes were photolyzed as described above. ATP depletions were effected by addition of glucose to a final concentration of 7 mM, then addition of 0.3 units of hexokinase, and incubation at 37 °C for 10 minutes. During the course of splicing, aliquots were removed at various times, the reactions in the aliquots were quenched by extraction with phenol/chloroform/isoamyl alcohol, the products were precipitated with ethanol, and then subjected to denaturing PAGE (15%; 29:1). Dried gels were exposed to a Molecular Dynamics phosphor screen which was then scanned using a Molecular Dynamics Storm 860 Phosphorimager.

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