Site-specific cross-linking of mammalian U5 snRNP to the 5' splice site before the first step of pre-mRNA splicing

Jacqueline R. Wyatt, 1,2 Erik J. Sontheimer, 2 and Joan A. Steitz

Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, Howard Hughes Medical Institute, Boyer Center for Molecular Medicine, New Haven, Connecticut 06536-0812 USA

We have used a site-specific cross-linking strategy to identify RNA and protein factors that interact with the 5' splice site region during mammalian pre-mRNA splicing. Two different pre-mRNA substrates were synthesized with a single 32P-labeled 4-thiouridine residue 2 nucleotides upstream of the 5' splice site. Selective photoactivation of the 4-thiouridine residue after incubation of either substrate under splicing conditions in HeLa nuclear extract resulted in cross-links to the U5 snRNA and the U5 snRNP protein p220. These ATP-dependent interactions occur before the first step of splicing. The U5 snRNA cross-links map to a phylogenetically invariant 9-nucleotide loop sequence and do not require Watson-Crick complementarity to the 5' exon. Cross-links of this position in the pre-mRNA to U1, but not to U2, U4, or U6 snRNAs, were also observed. The kinetics of U1 and U5 cross-link formation are similar, both peaking well before reaction intermediates appear.

[Key Words: Site-specific cross-linking, pre-mRNA splicing, U5 snRNP, 5' splice site, 4-thiouridine]

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Noncoding sequences called introns interrupt many eukaryotic genes and must be removed accurately from pre-messenger RNA (pre-mRNA) to yield mature mRNA. This splicing reaction occurs in two steps (for review, see Steitz et al. 1988; Green 1991; Guthrie 1991). The first step is cleavage at the 5' intron-exon junction concomitant with formation of a 2'-5' phosphodiester bond between the 5' end of the intron and a branchpoint nucleotide within the intron to generate a lariat intermediate. In the second step, the free 3' hydroxyl group of the 5' exon attacks the phosphodiester bond at the 3' splice site, resulting in ligation of the exons and release of the intron lariat. This splicing mechanism resembles that of the RNA-catalyzed self-splicing of group II introns found in fungal and plant mitochondria and plant chloroplasts (Guthrie 1991; Sharp 1991).

Nuclear pre-mRNA splicing in eukaryotic cells is mediated by a large complex, the spliceosome, which contains five small nuclear RNAs (snRNAs) [U1, U2, U4, U5, and U6 snRNAs] and numerous proteins (Reed 1990; for review, see Steitz et al. 1988; Green 1991; Guthrie 1991). Identification of RNA and protein factors that interact specifically with the pre-mRNA has been crucial to the understanding of the mechanisms of splice site selection and spliceosome assembly. Two snRNA/pre-mRNA interactions—base-pairing between U1 and the 5' splice site and between U2 and the branchpoint—have been intensively studied (for review, see Steitz et al. 1988; Green 1991; Guthrie 1991). Recently, additional snRNA/pre-mRNA interactions have been detected. Sawa and Shimura (1992) observed UV light-induced cross-links between U6 snRNA and both the 5' splice site and the branchpoint regions of pre-mRNA. Genetic suppression experiments in the yeast Schizosaccharomyces pombe indicated that U1 snRNA also base pairs to the invariant AG dinucleotide at the 3' splice site (Reich et al. 1992). Likewise, Newman and Norman (1991, 1992) used genetic suppression analyses to conclude that in Saccharomyces cervisiae U5 snRNA can base-pair with exon sequences at the 5' and 3' splice sites of certain mutant pre-mRNA substrates. Numerous interactions between protein factors and pre-mRNA have also been characterized. Several polypeptides have been reported to bind specifically to the polypyrimidine tract/3' splice site, including intron-binding protein (IBP) [Gerke and Steitz 1986; Tazi et al. 1986], polypyrimidine tract-binding protein [heterogenous nuclear ribonucleoprotein (hnRNP) 1/PTB] [Garcia-Blanco et al. 1989; Patton et al. 1991; Ghatti et al. 1992], U2 accessory factor [U2AF] [Zamore and Green 1989], and several hnRNP proteins [Swanson and Dreyfuss 1988]. The U5-specific protein product of the yeast PRP8 gene, and its mammalian analog p220, can be cross-linked to pre-mRNA [Garcia-Blanco et al. 1990; Whittaker and Beggs 1991].

1Present address: Isis Pharmaceuticals, Carlsbad Research Center, Carlsbad, California 92008 USA.
2These authors contributed equally to this work.
Stolow and Berget (1991) have implicated two proteins as 5' splice site-binding factors. The conclusion that the conserved loop sequence of U5 snRNA can base-pair with 5' exon sequences (Newman and Norman 1992) relied on the activation of cryptic 5' splice sites by mutant U5 snRNAs when the invariant G in the first position of the intron was changed to an A, abolishing splicing at the normal site. The proposed U5/pre-mRNA Watson–Crick base-pairing was surprising because of the extremely weak conservation of these exon sequences (Mount 1982) and the demonstration (Aebi et al. 1986) that positions -1 to -3 upstream of a mammalian 5' splice site can be mutated extensively without any adverse effect on splicing. Thus, it was not clear whether such an interaction would occur in the absence of mutations in the U5 loop and the 5' splice site, particularly if there were no Watson–Crick complementarity. Also, if contacts are formed during wild-type splicing, when do they occur relative to the well-characterized base-pairing of U1 snRNA with the 5' splice site (for review, see Steitz et al. 1988; Green 1991)? Are these interactions conserved in all eukaryotes? Do other components of the U5 snRNP or the spliceosome also contact 5' exon sequences? Answers to these questions are essential for understanding how the 5' splice site is defined and activated for the first step of the splicing reaction.

Here, we have exploited the recent development of a procedure for efficient synthesis of large RNA molecules with single modifications at defined sites (Moore and Sharp 1992) to introduce the cross-linking agent 4-thiouridine into pre-mRNA substrates near the 5' splice site. Using this strategy, we have shown that two different pre-mRNA substrates with a single 32P-labeled 4-thiouridine residue 2 nucleotides upstream of the 5' splice site can be cross-linked to U5 snRNP components before the first step of splicing in HeLa nuclear extracts.

## Results

### Synthesis of pre-mRNA substrates for site-specific cross-linking

Pre-mRNAs containing modified nucleotides at specific sites can be prepared efficiently by joining two in vitro-transcribed RNAs (5' and 3' fragments) using T4 DNA ligase in the presence of a bridging oligodeoxynucleotide (Moore and Sharp 1992). To synthesize splicing substrates containing a single radioactive phosphate adjacent to a photoactivatable group, transcription of the 5' fragment was primed by G(5')ppp(5')G while the 3' fragment was initiated with 4-thiouridyl-(3'-5')-guanosine [4-thioUpG]. The free 5' hydroxyl group of the 3' fragment was then phosphorylated with [γ-32P]-ATP using T4 polynucleotide kinase, and the two RNAs were ligated. The uridine analog 4-thiouridine has several advantages as a cross-linking agent: It can be photoactivated specifically and efficiently at a unique wavelength (>300 nm) to form cross-links to both RNA and protein by a free radical (Bergstrom and Leonard 1972; Dontsova et al. 1991; Lemaigre Dubreuil et al. 1991), its lack of bulk minimizes nonspecific steric interference; 4-thio-UpG is commercially available, and the half-life of the excited state after photoactivation within tRNA is on the order of microseconds (Favre 1990). RNA cross-links of 4-thiouridine are generally not to a base-paired partner but, rather, detect non-Watson–Crick interactions (Bergstrom and Leonard 1972; Lemaigre Dubreuil et al. 1991).

Two pre-mRNA substrates containing 32P-labeled 4-thioU located 2 nucleotides upstream of the 5' splice site were used in this study (Fig. 1). 32P-Labeled 4-thio Ad is a standard splicing substrate derived from the adenovirus major late transcription unit (Solnick 1985), except that the G residue 2 nucleotides upstream of the 5' splice site has been replaced by 4-thiouridine. 32P-4-thio SL/Ad contains the Leptomonas collosoma spliced leader RNA sequence connected in cis to the adenovirus 3' splice site (Bruzik and Steitz 1990). This latter substrate is efficiently spliced in extracts in which the 5' end of U1 snRNA has been cleaved by oligonucleotide-directed RNase H digestion (Bruzik and Steitz 1990), and was used to identify factors that interact with a 5' splice site that does not require U1 base-pairing.

32P-labeled 4-thio substrates are spliced in vitro and become cross-linked to U5 and U1 snRNAs

Although 4-thiouridine was introduced only 2 nucleotides upstream of the 5' splice site, this modification and the point mutation in the 32P-labeled 4-thio Ad substrate do not interfere with in vitro splicing. Figures 2B and 3B (lower panels) show that both 32P-labeled 4-thio SL/Ad and 32P-labeled 4-thio Ad are spliced with normal kinetics in HeLa nuclear extract, with the 5' exon intermediate and spliced product appearing after 15 or 20 min, respectively. Because the single radioactive phosphate lies within the 5' exon, lariat–3' exon intermediate...
Figure 2. Cross-linking of HeLa nuclear extract RNAs to $^{32}$P-labeled 4-thio SL/Ad during in vitro splicing. (A) Oligonucleotide-directed RNase H digestion of cross-linked RNAs. Oligonucleotides are designated at the top of each lane; the 4-thiouridine-dependent U1 and U5 cross-linked species are identified at left; the slowest migrating band [designated *] is a 4-thiouridine-independent U1 cross-linked species. (B) Splicing and cross-linking time course. (Top) A longer gel of RNAs taken from the splicing reaction below and cross-linked after the indicated number of min of incubation at 30°C [the pre-mRNA substrate has run off the bottom of the portion of the top gel shown]. [Lane 1 (input)] The pre-mRNA substrate; [lane 2 (no UV)] RNA from a non-irradiated sample; [lane 3 (no extract)] pre-mRNA incubated in the absence of nuclear extract; [lanes 4–12 (complete)] RNA from splicing reactions incubated for the indicated times [in min]; [lane 13 (no 4-thioU)] is a splicing reaction with a substrate that contains uridine at position −2 of the 5' exon rather than 4-thiothiouridine. The pre-mRNA spliced product and the 5' exon intermediate are identified at bottom right. (C) Cross-linking in the absence of ATP or magnesium. [Lane 1 (−ATP)] RNA from a splicing reaction lacking ATP and creatine phosphate; [lanes 2–10 (+EDTA)] RNA from splicing reactions containing 4 mM EDTA and no added MgCl$_2$. The 4-thiouridine-dependent U1 and U5 cross-linked species are indicated at right.

and lariat product are not visible in these autoradiographs. As expected from previous results (Bruzik and Steitz 1990), $^{32}$P-labeled 4-thio SL/Ad is spliced efficiently even after the 5' end of U1 snRNA is destroyed by oligonucleotide-directed RNase H cleavage, whereas splicing of $^{32}$P-labeled 4-thio Ad is inhibited by this treatment (data not shown).

To examine RNAs that become cross-linked to the 5' splice site of $^{32}$P-labeled 4-thio SL/Ad, aliquots were removed from the splicing reaction at various times, irradiated with 302 nm light for 2 min, digested with proteinase K, and analyzed by gel electrophoresis. Figure 2B (top) shows three slowly migrating bands [each representing 0.1–0.5% of the substrate] that are dependent on UV light and nuclear extract (cf. lane 9 with lanes 2 and 3). Only the slowest band forms with a control substrate synthesized using a UpG primer for the 3' fragment [Fig. 2B, lane 13]; this demonstrates that the two faster migrating species are cross-linked by the single 4-thiouridine residue, because the addition of a single sulfur atom at position −2 relative to the 5' splice site would not be expected to cause photoactivation of any other nucleotides in the pre-mRNA. Because the slowest migrating cross-linked band appears in the absence of 4-thiouridine

Figure 3. Cross-linking of HeLa nuclear extract RNAs to $^{32}$P-labeled 4-thio Ad during in vitro splicing. (A–C) are exactly as in Fig. 2, except that the $^{32}$P-labeled 4-thio Ad substrate was used.
Mammalian U5 snRNP/5’ splice site interactions

[Fig. 2B, lane 13], it is not necessarily cross-linked at the 5’ splice site. All three species are directly immunoprecipitated by antibodies against the trimethylguanosine cap found on all splicesomal snRNAs except U6 [Krainer 1988] [data not shown]. The snRNAs cross-linked to the substrate by 4-thiouridine were identified as U5 and U1 by specific RNase H cleavage in the presence of complementary oligonucleotides [Fig. 2A]; un-cross-linked 32P-P-labeled 4-thio SL/Ad is not digested during this reaction [data not shown]. The snRNA cross-linked in the absence of 4-thiouridine was also shown to be U1 (* in Fig. 2). We were not able to identify any RNAs cross-linked to the cleaved 5’ exon intermediate; such a cross-linked species would be likely to migrate in the same region of the gel as the pre-mRNA substrate or spliced product and would therefore be obscured.

Kinetic analysis of cross-link formation in the same splicing reaction shown in Figure 2B [bottom] showed that the U1/5’ splice site interaction with the SL/Ad substrate occurs without incubation at 30°C [Fig. 2B, lane 4, upper panel], consistent with earlier studies [Black et al. 1985; Bindereif and Green 1987; Michaud and Reed 1991]. The yield of U1/pre-mRNA cross-link increases after 1 min of incubation at 30°C and decreases steadily thereafter [Fig. 2B, lanes 4–12, top]. In contrast, the U5/5’ splice site interaction is not detected after 0 or 1 min of incubation but appears strongly at 5 min and diminishes as splicing proceeds [Fig. 2B, lanes 4–12, top]. The slightly delayed appearance of the U5 relative to the U1 snRNA cross-link is consistent with previous results showing that incorporation of the U5 snRNP into the spliceosome occurs after the binding of U1 and U2 snRNPs to the substrate [for review, see Steitz et al. 1988; Green 1991]. Because splicing intermediates and products do not appear until 15 min of incubation in this reaction mixture [Fig. 2B, lanes 4–12, bottom], the U5/5’ splice site interaction precedes the first step of splicing. No additional cross-linked bands appear at subsequent stages of the reaction, indicating that no other RNA species contact position −2 of the 5’ exon before the first step of splicing.

Incubation of 32P-labeled 4-thio SL/Ad in nuclear extracts in the absence of ATP allows the U1/5’ splice site cross-link to occur but blocks the U5 interaction at all time points [Fig. 2C, lane 1; data not shown], in agreement with the previously described ATP requirement for U4/U5/U6 tri-snRNP assembly into the spliceosome [Bindereif and Green 1987; Konarska and Sharp 1987]. Incubation in the presence of EDTA allows spliceosome assembly but blocks both steps of splicing [Reed et al. 1988]. Under these conditions [Fig. 2C, lanes 2–10], both the U1 and U5 cross-links are formed [albeit with significantly slower kinetics], but no splicing products or intermediates are observed [data not shown]. The U5/5’ splice site cross-link does not diminish with time, suggesting that in the absence of magnesium, the U5 snRNP remains in association with the 5’ exon. Furthermore, none of the U1 or U5 snRNA cross-links form during incubation in S-100 extracts [data not shown], which contain all of the snRNPs and protein factors required for splicing except for splicing factor 2 [SF2] at approximately the same concentration as nuclear extract [Krainer et al. 1990]. The lack of U5 cross-linking in the absence of either ATP or SF2 indicates that detection of the U5/5’ exon interaction is restricted to conditions that allow spliceosome assembly, and does not reflect nonspecific interactions between free U5 snRNP and pre-mRNA.

Comparable analyses using the 32P-labeled 4-thio Ad splicing substrate [Fig. 3] likewise revealed an ATP-dependent U5 cross-link, which first appears after 5 min of incubation [before the first step of splicing] and accumulates in the presence of EDTA. Thus, interactions between the 5’ exon and U5 snRNA are not restricted to the SL-containing substrate. With the 32P-labeled 4-thio Ad pre-mRNA, two different U1/5’ splice site cross-linked species are seen [Fig. 3A,B]; both form immediately at 0°C [Fig. 3B, lane 4] and in the absence of either ATP or magnesium [Fig. 3C]. An additional 4-thiouridine-independent U1 cross-link [labeled *] is again observed. These cross-links to U1 snRNA are being analyzed further.

The conserved loop of U5 snRNA cross-links to 5’ exon sequences

To identify the region of U5 snRNA that contacts the 5’ exon, the cross-linked site was identified by primer extension blockage [Fig. 4A,B]. Reverse transcription was performed on gel-purified U5/pre-mRNA cross-linked species using a primer complementary to nucleotides 84–104 of U5. For both the 32P-labeled 4-thio SL/Ad and the 32P-labeled 4-thio Ad substrates, comparison of the blockage sites [lanes 8] with a sequencing ladder [lanes 2–6] revealed extension stops in four positions, each of which is 1 nucleotide 3’ to 1 of the 4 uridine residues in the conserved loop of U5 snRNA [Fig. 4C]. The strongest stop indicates cross-linking to U41, the nucleotide proposed by Newman and Norman (1992) to base-pair with position −2 relative to the 5’ splice site. Figure 4, A and B, shows that reverse transcription is not blocked at these sites when purified U5 snRNA is used as a template [lanes 7], nor does blockage result from fortuitously comigrating RNAs present in the splicing reaction [lanes 6] or the pre-mRNA substrate [lanes 11] acting as template. Furthermore, these stops are not the result of nonspecific UV damage, as they are not generated with analysis of RNA from extracts irradiated in the absence of substrate [lanes 10].

A U5 snRNP protein cross-links to the 5’ exon

Treatment of irradiated splicing reactions with nuclease P1 rather than protease allows the analysis of proteins cross-linked through 4-thiouridine. Cross-linked proteins become radioactively labeled by transfer of the 32P-labeled phosphate [RNase P1 yields 5’ nucleoside monophosphates [Fujimoto et al. 1974] and can be visualized by gel electrophoresis and autoradiography. Because numerous proteins present in nuclear extract bind RNA in
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Figure 4. Sites of U5 snRNA cross-linking to 4-thiouridine located 2 nucleotides upstream of the 5' splice site. (A, B) Primer extension mapping of cross-links to 32P-labeled 4-thio SL/Ad and 32P-labeled 4-thio Ad, respectively, using an oligonucleotide complementary to nucleotides 84-104 of U5 RNA. [Lanes 1 (M)] 32P-Labeled pBR322 MspI DNA markers [sizes in nucleotides indicated at right of each panel]; [Lanes 2-5] dideoxynucleotide sequencing ladders of U5 snRNA; [lane 6 (blank)] a reaction without dideoxynucleotide. Reverse transcription templates prepared as described in Materials and methods are indicated at the top of lanes 7-11. The positions of the full-length primer extension product and the invariant 9-nucleotide loop are shown at left of each panel. (C) Secondary structure of U5 snRNA. The evolutionarily invariant 9-nucleotide loop sequence is in outlined letters; subscript m indicates 2'-O-methyl modifications, and G indicates a pseudouridine residue. Sites of cross-linking are designated by arrows; the size of the arrow indicates the relative efficiency of cross-linking. Nucleotides are numbered from the 5' end of U5 RNA.

To determine the identities of the cross-linked proteins, extensive RNase T1 digestion was performed before the addition of antibodies; RNase digestion disrupts a non-sequence-specific manner [Fig. 5, lane 2], splicing complexes were selected by immunoprecipitation with various snRNP-specific antibodies before analysis.

Proteins cross-linked to 32P-labeled 4-thio SL/Ad are shown in Figure 5. Although multiple bands are present in the total sample [Fig. 5A, lane 2], immunoprecipitation with anti-Sm monoclonal antibody [lanes 3-7; Lerner et al. 1981] or Laj anti-(U5)RNP patient serum [lanes 8-11; Sontheimer and Steitz 1992; Y. Okano, J. Craft, and T. Medsgcer, in prep.] simplifies the pattern considerably. Two strong bands of 16 and 70 kD are present in the anti-Sm precipitate at all times [lanes 3-7]. An additional protein of ~220 kD appears after 5 min of incubation [lanes 5-7], with similar kinetics to the U5 snRNA/5' exon cross-link. These three proteins are also present after 5 min of incubation in the Laj-precipitated samples [lanes 9-11]. Another protein present at early times, but only in the anti-Sm precipitate [lanes 3-5], migrates with an apparent molecular mass of 62 kD, similar to that of a previously described putative splicing factor called hnrNIP I/PTB [Gil et al. 1991; Patton et al. 1991; Ghetti et al. 1992]. The same set of proteins were seen using several different active nuclear extracts prepared under different final dialysis conditions (data not shown), indicating that the protein cross-linking profile does not depend on a particular concentration of KCl or (NH4)2SO4. No cross-linked proteins were observed when a control substrate lacking 4-thiouridine was used (data not shown).
the spliceosome and minimizes indirect immunoprecipitation of nonantigenic proteins present in a multicomponent complex. In Figure 5B, the control nonimmune serum precipitates no proteins either before or after RNase treatment, as expected [lanes 3,4]. The cross-linked 220-kD protein is precipitated efficiently by Laj anti-(U5)RNP patient serum either with or without prior RNase treatment [lanes 7,8], arguing strongly that this polypeptide is a U5 snRNP protein. It is likely to be the previously identified protein p220 present in purified U5 snRNPs, U4/U5/U6 tri-snRNPs, and spliceosomes [Bach et al. 1989; Pinto and Steitz 1989; Reed 1990; Behrens and Luhrmann 1991], as p220 and its yeast analog, PRP8, are of similar size and are known to contact the pre-mRNA (Garcia-Blanco et al. 1990; Whittaker and Beggs 1991). p220 is not precipitated by anti-[U1]RNP serum before or after RNase digestion [data not shown]. The specificity of the p220/5' exon interaction was further established by competition experiments: p220 cross-linking to 32P-labeled 4-thio SL/Ad was abolished in the presence of a 100-fold excess of unlabeled splicing substrate, whereas other protein cross-links remained; competition with nonspecific RNAs did not block p220 cross-linking [data not shown]. The amount of 16-kD protein immunoprecipitated by Laj varied between experiments [cf. Fig. 5B, lane 7, with Fig. 5A, lane 10].

After RNase treatment, all cross-linked species except the prominent 16-kD protein are greatly diminished in the anti-Sm precipitate [Fig. 5B, cf. lane 6 with lane 5]. This shows that the 16-kD protein possesses an Sm epitope; it is likely to be a core Sm D protein [Pettersson et al. 1984; Lehmeier et al. 1990] as the D polypeptides are the only proteins of this size that react with the Y-12 monoclonal antibody [Lehmeier et al. 1990]. The 16-kD protein is also selected by anti-[U1]RNP immunoprecipitation in the absence of prior RNase treatment [data not shown], indicating that it may be a core protein associated with the U1 snRNP. The ~70-kD cross-linked protein [Fig. 5A, lanes 2–7, 9–11] does not appear to be the U1-specific 70K protein or IBP, as it is not strongly immunoprecipitated from RNase-treated reactions by anti-U1[RNP] patient sera [data not shown] or by Y12 anti-Sm monoclonal antibodies [Fig. 5B, lane 6], which recognize U1 70K and IBP, respectively.

The 62-kD protein [Fig. 5A, lanes 3–7] was identified as hnRNP I/PTB by immunoprecipitation after RNase T1 digestion. Figure 5B [lanes 11, 13] shows that 7G12 anti-hnRNP I monoclonal antibodies [a gift from S. Piñol-Roma and G. Dreyfuss, University of Pennsylvania, Philadelphia] and affinity-purified anti-PTB polyclonal antibodies [a gift from A. Gil and P. Sharp, Massachusetts Institute of Technology, Cambridge, MA] precipitate the 62-kD protein efficiently, whereas purified IgG from the anti-PTB preimmune serum [lane 12] and sec-

**Figure 5.** Cross-linking of HeLa nuclear extract proteins to 32P-labeled 4-thio SL/Ad during in vitro splicing. (A) Time course of protein cross-linking after anti-Sm and anti-[U5]RNP selection. Numbers at the top of lanes 2–11 are minutes of incubation at 30°C. [O (upper right)] is the boundary between the stacking and separating portions of the gel. [Lane 1 (M)] 14C-labeled protein markers; sizes [in kD] are given at left; [Lane 2 (no ppt.)] is a sample that was not subjected to immunoprecipitation. The antibodies used for immunoprecipitation in lanes 3-11 are given at the top. [Lanes 2 (no ppt.)] is a sample that was not subjected to immunoprecipitation. The bands identified as p220, PTB, and Sm D core protein are indicated between the panels. (B) Immunoprecipitation of cross-linked proteins after RNase T1 digestion. O, M, and protein identifications are as in A. [Lane 2 (no precip.)] A sample that was not subjected to immunoprecipitation; the antibodies used for immunoprecipitation are given at the top. [Lanes 9–11 (2~ Rabbit anti-mouse IgG antibody; [lane 11 (7G12)] anti-hnRNP I monoclonal antibody; [lane 10 (SP2/0)] ascites fluid from the parental myeloma used to generate the 7G12 hybridoma; [lane 13 (A.P. anti-PTB)] affinity-purified anti-PTB antibodies, [lane 12 (preimmune IgG)] affinity-purified IgG from the preimmune serum. Samples were incubated for 2 (lanes 9–13) or 15 (lanes 2–8) min at 30°C before cross-linking. (+ or -) The presence or absence of RNase T1 digestion before immunoprecipitation.
ondary antibody alone (lane 9) or in conjunction with ascites fluid from the SP2/0 parental myeloma (lane 10) do not.

Protein cross-linking experiments with ³²P-labeled 4-thio Ad gave similar results (Fig. 6), although the number of prominent bands seen at all times was higher (lanes 3–7). The onset of p220 cross-linking was slower (lanes 6, 7, 11, 12), mimicking the slightly slower kinetics of splicing of this substrate compared with ³²P-labeled 4-thio SL/Ad (Figs. 2B and 3B). The 62-kD protein (lanes 3–7) cross-linked to this substrate was shown to be hnRNP I/PTB by immunoprecipitation analyses, as described above (data not shown).

*p220 contacts the 5' exon in the spliceosome*

Electrophoresis of cross-linked ³²P-labeled 4-thio SL/Ad splicing reactions in nondenaturing gels (Konarska and Sharp 1986) separates spliceosomes [B and C complexes] from prespliceosomes [A complex] and non-specific H complex [Fig. 7A]. These complexes have the same mobility as those produced in a nonirradiated sample [data not shown]. Formation of the B complex occurs within 5 min of incubation [lane 2], in parallel with U5 snRNA (Fig. 2) and p220 (Fig. 5) cross-linking. RNase T1 treatment of an excised complex gel lane, followed by protein gel electrophoresis in the second dimension, reveals that the 220-kD protein is observed in the B/C complex (Fig. 7B). Thus, the cross-link between the 220-kD protein and the 5' exon occurs in the spliceosome. Because the U5 snRNP is present in spliceosomes but not in prespliceosomes or hnRNP complexes (for review, see Steitz et al. 1988; Green 1991) and because p220 is the only protein in its size range that can be UV-cross-linked to pre-mRNA in purified spliceosomes (D. Staknis and R. Reed, pers. comm.), further evidence is provided that the cross-linked protein is the U5-specific p220 protein. Cross-linked Sm D core protein is observed predominantly in the A complex, whereas a number of other proteins, including one of ~62 kD [perhaps hnRNP I/PTB], appear primarily in the H complex.

**Figure 6.** Cross-linking of HeLa nuclear extract proteins to ³²P-labeled 4-thio Ad during in vitro splicing. All designations are as in Fig. 5A.

**Discussion**

Specific contacts between trans-acting factors and the pre-mRNA are likely to underlie the juxtaposition of distant splice sites and the subsequent catalytic reactions of splicing. Here, we have developed a powerful 4-thiouridine-based site-specific cross-linking system to identify both RNA–RNA and RNA–protein contacts during splicing in vitro. Using commercially available 4-thio-UPG and the ligation technique developed by Moore and Sharp (1992), we introduced a single photocleavable nucleotide at position −2 relative to the 5' splice site of the adenovirus splicing substrate and an analogous SL RNA-containing substrate. After short [1–2 min] irradiation with 302 nm light at various times during the splicing reaction, interactions between this site and the U1 and U5 RNAs and multiple proteins including p220 [a U5-specific polypeptide], hnRNP I/PTB, and Sm D are detected. The kinetics suggest that contacts between the 5' exon and the U5 and U1 RNAs occur very early, before the first step of splicing. Given the promiscuity of 4-thiouridine cross-linking (Bergstrom and Leonard 1972; Favre 1990; Lemaigre Dubreuil et al. 1991), the absence of cross-links to U2, U4, and U6 suggests that these other spliceosomal snRNAs do not interact directly with the 5' exon before the first step of splicing.

The U5 snRNP/5' exon interactions that we have characterized occur in a mammalian system in the absence of mutations affecting invariant residues in either the pre-mRNA substrate or in U5 snRNA and are in excellent agreement with yeast genetic analyses [Newman and Norman 1992]. Moreover, the U5 contacts are observed in two different substrates with distinct 5' splice sites, one of which is actively spliced in the absence of the 5' end of U1 RNA [Bruzik and Steitz 1990]. The U5/5' exon contacts occur in the spliceosome, concomitant with or immediately after the incorporation of U5 snRNP into the splicing complex and then diminish during the splicing reaction; nonspecific interactions would simply accumulate with time. The cross-links require both ATP and the non-snRNP protein SF2 [Krainer et al. 1990], and are therefore not the result of nonspecific-binding properties of free U5 snRNP. Furthermore, p220 cross-linking is specifically lost in the presence of excess competitor splicing substrate. The above criteria all argue that a U5/5' exon interaction, as originally proposed by Newman and Norman [1992], is general and functionally significant.

In U5 snRNA, the cross-links are localized to 4 adjacent nucleotides within a phylogenetically invariant loop sequence. This region is available for base-pairing within the U5 snRNP particle [Black and Pinto 1989; Lamm et al. 1991] but does not exhibit extensive Watson–Crick complementarity to 5' exon sequences (e.g., for the SL/Ad substrate, one G-U and two U-U "pairs" would be formed). Because the 4-thiouridine cross-links are indicative of non-Watson–Crick interactions [Lemaigre Dubreuil et al. 1991], no rigid constraints are placed on exon sequences [Mount 1982], allowing more evolutionary flexibility in the coding region. Such non-
standard pairing interactions with nucleotides upstream of the 5′ splice site are probably facilitated by the abundance of pyrimidines in the conserved loop of U5 snRNA, as suggested by Newman and Norman (1992). The U5/5′ exon contacts mimic a conserved base-pairing interaction in group II self-splicing introns (Jacquier 1990) and therefore provide additional evidence of parallels between the spliceosome and group II introns.

Our observation that the amount of cross-linked U5 snRNA begins to diminish before splicing proceeds indicates that the conserved loop of U5 snRNA may shift to allow other interactions before 5′ splice site cleavage. This is consistent with a proposal for base-pairing between U5 and intron sequences at the 5′ splice site, based on the characterization of a U5/pre-mRNA psoralen cross-link [Wassarman and Steitz 1992] that appears with delayed kinetics relative to the U5/5′ exon contact that we have observed. In contrast, the amount of cross-linking of the large, U5 snRNP protein p220 does not diminish significantly as the reaction progresses (Figs. 5 and 6; data not shown). Initially, p220 may assist in stabilizing the relatively weak U5 snRNA/5′ exon non-Watson–Crick interactions. Later, p220 can be considered an excellent candidate for the factor that holds the cleaved 5′ exon intermediate in the spliceosome after 5′ splice site cleavage and before exon ligation, U5 snRNA probably does not perform this role based on its subsequent interaction with intron sequences before 5′ splice site cleavage [Wassarman and Steitz 1992]. p220 is the mammalian homolog of the yeast PRP8 protein (Lossky et al. 1987; Anderson et al. 1989; Pinto and Steitz 1989). Both p220 and PRP8 were reported previously to associate with the pre-mRNA during splicing [García-Blanco et al. 1990, Whittaker and Beggs 1991], but the sites of interaction were not defined in these studies. PRP8 interacts genetically with the putative RNA helicases PRP28 [Strauss and Guthrie 1991] and SPP81 [Jamieson et al. 1991], either of which could function to promote an ATP-dependent transition between U5/5′ exon and U5/intron interactions.

In addition to cross-links to U5 snRNA, we have observed cross-links between the 5′ splice site and U1 RNA. These have not yet been mapped within the U1 molecule, but it is interesting that two different U1/5′ splice site cross-links were observed with the 32P-labeled 4-thio Ad substrate and only one with 32P-labeled 4-thio SL/Ad. Cross-linking of the latter substrate to U1 was not anticipated, as SL RNA-containing substrates [including 32P-labeled 4-thio SL/Ad (data not shown)] are spliced even after destruction of the 5′ end of U1 snRNA by oligonucleotide-directed RNase H cleavage (Bruzik and Steitz 1990). The formation of a U1/5′ splice site cross-link implies that the 32P-labeled 4-thio SL/Ad substrate nonetheless contacts U1 snRNA in untreated extracts. This observation is in accord with the results of 2′-O-methyl oligoribonucleotide inhibition and psoralen cross-linking experiments, which also suggest that the U1 snRNP does participate in the splicing of SL-containing pre-mRNAs even in the absence of U1/5′ splice site base-pairing (S. Serwert and J.A. Steitz, in prep.). Because the kinetic profiles of the U1 and U5 cross-links overlap [Figs. 2 and 3], it is conceivable that the 5′ splice site region of a single pre-mRNA interacts simultaneously with U1 and U5 snRNAs in the newly assembled spliceosome (Steitz 1992).

In principle, site-specific incorporation of a photoactivatable group into an RNA substrate will be useful in the identification of interacting components in any biochemical process that proceeds in vitro (e.g., translation, polyadenylation, tRNA and rRNA processing, histone mRNA 3′ end formation, SRP-mediated protein translocation, group I–group II self-splicing, and replication of RNA viruses). An advantage of this experimental system is that it allows detailed kinetic analyses of both RNA–
RNA and RNA–protein contacts during the course of the reaction. The use of photoactivatable groups other than 4-thiouridine could increase the versatility of this approach by eliminating the requirement for a UG sequence in the RNA substrate.

**Materials and methods**

**DNA templates**

DNA oligonucleotides were synthesized by Peter Davis using Expeptides [Milligen/Biossearch] or John Flory using standard amidites [Yale University]. The template for synthesis of the 3’ SL/Ad fragment was made by oligonucleotide-directed mutagenesis of the construct L/Ad, described previously by Bruzik and Steitz [1990], using standard cloning methodologies. Briefly, the first 43 nucleotides of the L/Ad DNA-coding region and the BamHI site at the 3’ end of the coding region were deleted, and a BamHI site was inserted within the intron between the spliced leader sequences and the adenovirus sequences. The EcoRI-HindIII fragment was subcloned into the EcoRI–HindIII sites of pUC118, and the plasmid was digested with SalI before transcription. The 3’ Ad template was generated by polymerase chain reaction (PCR) using pSPAd plasmid [Solnick 1985] as template; primers were as follows: 5’-GCCGCAGCTTCTCAATACGACCTCATAAGTAGATGACCTCTCTTCA-3’ and 5’-GGCCCAAGCTTGGCTGACGTTAACATCCAGCGCGTGGTTCGG-3’. The resulting 363-nucleotide PCR product contained a bacteriophage T7 RNA polymerase promoter, 1 nucleotide of 5’ exon, the 243-nucleotide intron, and a 90-nucleotide half RNA (4 μM and 6 μM final concentrations, respectively), and distilled water (17 μl, final volume), the reactions were heated to 90°C for 2 min and cooled at room temperature for 5–10 min. One microliter of ligase buffer [0.5 mM Tris-HCl (pH 7.5), 10 mM ATP, 10 mM MgCl₂, and 1 μg/μl of acetylated bovine serum albumin (BSA)] [New England Biolabs], 0.4 μl of 1 M DTT, and 2 μl of 10 U/μl T4 DNA ligase [U.S. Biochemical] were added; after a 1-hr incubation at 37°C, the reaction was reheated to 90°C for 2 min and cooled at room temperature for 5–10 min. An additional 2 μl of 10 U/μl T4 DNA ligase was added, and the reaction was again incubated at 37°C for 1 hr. Samples were PCA extracted, ethanol precipitated, purified by electrophoresis in a 6% polyacrylamide/8 M urea sequencing gel, and eluted in 0.4 ml of elution buffer [0.5 M sodium acetate, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% SDS, and 25 μg/ml of total yeast RNA] by mixing overnight at room temperature. Eluted RNAs were ethanol precipitated and resuspended in distilled water. Yields in the ligation reaction were ~50% for 32P-labeled 4-thio SL/Ad and 10–15% for 32P-labeled 4-thio Ad.

**RNA substrates**

All RNAs were synthesized by in vitro transcription in 0.25-ml volumes at 37°C for 1.5 hr. 5’ SL/Ad and 5’ Ad transcription reactions contained 40 μM Tris-HCl [pH 8.0], 1 mM spermidine, 5 mM DTT, 8% polyethylene glycol, 5 mM G5’[ppp]5’[G] (Pharmacia), 1.5 mM each ribonucleoside triphosphate, 14 mM MgCl₂, 0.3 μM DNA template (see above), and 25 U/μl of T7 RNA polymerase. 3’ SL/Ad transcription reactions contained 40 μM Tris-HCl [pH 8.0], 1 mM spermidine, 5 mM DTT, 10 mM MgCl₂, 2 μM 4-thioUpG [Sigma] or uridylyl[3’-5’]guanosine [UpG; Sigma], 1 mM each ribonucleoside triphosphate, 0.1 μg/μl of DNA template, and 4 U/μl of T7 RNA polymerase. 3’ Ad transcription reactions contained 40 μM Tris-HCl [pH 8.0], 4 mM spermidine, 10 mM DTT, 20 mM MgCl₂, 5 μM each ribonucleoside triphosphate, 0.2 μg/μl of DNA template, 10 μM 4-thio-UpG or UpG, and 25 U/μl of T7 RNA polymerase. Reactions were stopped by the addition of EDTA to a final concentration of 15 mM, extracted with phenol/chloroform/isoamyl alcohol (50 : 49 : 1) [PCA], ethanol precipitated, and purified by electrophoresis in a 10% polyacrylamide 8 μm urea sequencing gel. Concentrations of the purified RNA transcripts were determined by absorbance at 260 nm, using extinction coefficients determined as in Puglisi and Tinoco [1990].

The splicing substrates were synthesized by ligation of the 5’ and 3’ T7 RNA polymerase transcripts. The 3’ fragment was 5’-kinased using [γ-32P]-ATP. The two transcripts were annealed to a DNA-bridging oligodeoxynucleotide complementary to 15–18 nucleotides at the 3’ end of the 5’ fragment and 15–18 nucleotides at the 5’ end of the 3’ fragment and ligated using T4 DNA ligase [Milligan and Uhlenbeck 1989] are not used as ligation acceptors, furthermore, any 3’ fragment transcripts that fail to prime with 4-thio-UpG will not act as substrates for either kinase or ligase enzymes. Sequences of bridge oligonucleotides were 5’-AGTGAGTCGTATTA-3’ for the 32P-labeled 4-thio SL/Ad and 5’-GAGAGGGAGTACTCACCACAAACAGCTGGCCCTCGC-3’ for 32P-labeled 4-thio SL/Ad and 5’-GAGAGGGAGTACTCACCACAAACAGCTGGCCCTCGC-3’. The splicing reactions were irradiated on a parafilm-covered aluminum block on ice for 2 min at 302 nm with a UV transilluminator [model TM-15, UVP Inc.]. The sample was 2 cm from...
the light source, giving ~7000 μW/cm² of radiation. Samples were then incubated in 1.5 mg/ml of proteinase K (Beckman) and 0.1% SDS at 37°C for 20 min, PCA extracted, and ethanol precipitated. Splicing intermediates and products were visualized by autoradiography after gel electrophoresis on 15-cm, 8% polyacrylamide/8 M urea gels in 1 x TBE (0.1 M boric acid, 0.1 M Tris base, and 2 mM EDTA). For analysis of cross-linked RNAs, 32P-labeled 4-thio SL/Ad samples were subjected to electrophoresis on 36-cm, 8% polyacrylamide/8 M urea/1 x TBE gels such that the xylene cyanol marker dye was run 28 cm off the bottom of the gel. 32p-labeled 4-thio Ad samples were electrophoresed on 36 cm, 5% polyacrylamide/8 M urea/1 x TBE gels until the xylene cyanol was run 22 cm off the bottom of the gel. Cross-linked RNAs were visualized by autoradiography.

For RNase H reactions, deproteinized cross-linked RNAs were prepared after 1 hr of incubation in the presence of EDTA under splicing conditions described above and were then incubated for 1 hr at 37°C in a 10 μl reaction volume containing 40 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 20 ng/μl of carrier RNA, 200 ng/μl of oligodeoxynucleotide, and 0.3 U/μl of RNase H (Boehringer Mannheim). U₅45–75 oligonucleotide is 5'-CGGAGTGCAATG-3', and U₅68–88 oligonucleotide is 5'-CGGAGTGCAATG-3'. Samples were then incubated in 1.5 mg/ml of proteinase K [Beckman] and the beads were incubated at 45°C for 30 min. Six microliters of 5x SDS loading buffer [0.314 M Tris [pH 6.8], 50% glycerol, 0.05% SDS, 0.7 M β-mercaptoethanol, 0.025 mg/ml of bromophenol blue] were added; samples were heated to 95°C for 5 min, subjected to electrophoresis on a 14% polyacrylamide–SDS gel, and visualized by autoradiography. Alternatively, 60 units of RNase T1 was added after cross-linking but before immunoprecipitation; the samples were incubated at 45°C for 30 min, added to PAS-bound antibodies, and processed as described above except that immunoprecipitated samples were washed four times with 1 ml of IPP [0.5 M NaCl, 0.05% NP-40, and 50 mM Tris-HCl [pH 7.5]] and once with NET-2. For analysis of hnRNP 1/PTB, immunoprecipitations were performed with 100 ng of preimmune antibodies, 100 ng of affinity-purified anti-PTB antibodies, or 3.75 μg of secondary antibody with or without 10 μl of 7G12 or SP2/0 ascites fluid.

**Primer extension mapping of U5 cross-links**

Deproteinized cross-linked RNAs were prepared as described above after a 5-min 0.4-ml standard splicing reaction for 32P-labeled 4-thio SL/Ad, or a 1 hr 0.4-ml splicing reaction in the presence of 4 μM EDTA for 32P-labeled 4-thio Ad, using 6 x 10⁶ cpm of substrate. Control reactions without substrate were carried out in parallel. Samples were electrophoresed as described above and visualized by autoradiography; U5 cross-linked species (identified by electrophoretic mobility) and comigrating RNAs from the control reaction were cut out of the gel, electroeluted overnight at 150 V in 1 x TBE in an Elutrap (Schleicher & Schuell) according to the manufacturer's protocol, and ethanol precipitated. Total UV-treated extract RNA was prepared as in the control reactions except that the gel purification step was omitted. Gel-purified uncross-linked U5 RNA was prepared from anti-Sm-immunoprecipitated RNA by elution from a 10% polyacrylamide/8 M urea/1 x TBE gel after electrophoresis. Reverse transcription reactions using these RNA samples as template were carried out with an oligonucleotide primer complementary to nucleotides 84–104 of U5 snRNA as described by Black and Pinto (1989), digested with 2 μg/ml of RNase A at 37°C for 20 min, PCA extracted, and ethanol precipitated. Sequencing ladders were generated with the same U5 primer according to Zaug et al. (1984), purified using uncross-linked U5 snRNA as template. Samples were visualized by autoradiography after electrophoresis on 36-cm, 12.5% polyacrylamide/8 μM urea/1 x TBE gels such that the bromphenol blue marker dye was run 8 cm off the bottom of the gel.

**Protein–RNA cross-linking**

Y-12 anti-Sm monoclonal antibodies were prepared as described by Lerner et al. (1981), and La anti-US[snRNP] patient serum was provided by Y. Okano (Nihon Koken Hospital, Tokyo, Japan). Nonimmune serum was obtained from a healthy volunteer. 7G12 anti-hnRNP I monoclonal antibody and SP2/0 myeloma ascites fluid was obtained from S. Piñol-Roma and G. Dreyfuss (University of Pennsylvania, Philadelphia). Affinity-purified rabbit anti-mouse IgG secondary antibody was purchased from Zymed. Affinity-purified preimmune and anti-PTB antibodies were obtained from A. Gil and P. Sharp (MIT, Cambridge, MA).

Ten microliters of Y-12, La, or nonimmune antibodies was mixed with 2.5 μg of protein A–Sepharose [PAS] (Pharmacia) in 0.5 ml of NET-2 [150 mM NaCl, 0.05% NP-40, and 50 mM Tris-HCl [pH 7.5]] for 1 hr at room temperature and washed four times with 1 ml of NET-2. Splicing reactions incubated for the indicated times were cross-linked as described above (except that irradiation was for 1 min) and added directly to the PAS-bound antibodies. After mixing at 4°C for 30 min, samples were washed four times with 1 ml of NET-2, after all but ~20 μl of the final wash was removed, 60 units of RNase T1 (CalBiochem) and 1 unit of nuclelease P1 (Boehringer Mannheim) were added, and the beads were incubated at 45°C for 30 min. Six microliters of 5x SDS loading buffer [0.314 M Tris [pH 6.8], 50% glycerol, 0.05% SDS, 0.7 M β-mercaptoethanol, 0.025 mg/ml of bromophenol blue] were added; samples were heated to 95°C for 5 min, subjected to electrophoresis on a 14% polyacrylamide–SDS gel, and visualized by autoradiography. Alternatively, 60 units of RNase T1 was added after cross-linking but before immunoprecipitation; the samples were incubated at 45°C for 30 min, added to PAS-bound antibodies, and processed as described above except that immunoprecipitated samples were washed four times with 1 ml of IPP [0.5 M NaCl, 0.05% NP-40, and 50 mM Tris-HCl [pH 7.5]] and once with NET-2. For analysis of hnRNP 1/PTB, immunoprecipitations were performed with 100 ng of preimmune antibodies, 100 ng of affinity-purified anti-PTB antibodies, or 3.75 μg of secondary antibody with or without 10 μl of 7G12 or SP2/0 ascites fluid.

**Splicing complex analysis**

After incubation of splicing reactions at 30°C for the indicated times, 4-μl aliquots were cross-linked as described above, incubated on ice for 10 min in the presence of 0.5 μg/μl of heparin, and loaded directly on 4% polyacrylamide native gel [80:1 acrylamide/bis-acrylamide in 50 mM glycine/50 mM Tris base [pH 8.8]]. Electrophoresis was carried out at 350 V for 3 hr, complexes were visualized by autoradiography. For two-dimensional analysis, a complex gel lane of a 30-min splicing reaction was excised and incubated in 0.6 U/μl of RNase T1 for 1 hr at room temperature, followed by incubation in 5x SDS loading buffer for 1 hr at room temperature. The gel slice was then placed on top of the stacking portion of a 15% polyacrylamide–SDS gel, sealed to the gel with 1% agarose, and subjected to electrophoresis. Cross-linked proteins were visualized by autoradiography.

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J R Wyatt, E J Sontheimer and J A Steitz

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